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Medical Frontiers:
Confronting Alzheimer's

Promising Vaccine Targets Ravager of Minds

By Susan Okie
Washington Post Staff Writer
Tuesday, May 8, 2001; Page A01

TAMPA -- The moment he hits the cool water of the laboratory's baby pool, the brown mouse swims for dear life. He is 17 months old -- elderly for a mouse -- but he seems to have his goal in mind. He paddles to the center of the daisy-shaped water maze, looks around, then heads rapidly down the correct aisle and clambers to safety on an invisible platform.

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An aging mouse's successful navigation of a maze might seem unconnected to the plight of the estimated 4 million Americans who have Alzheimer's disease, a common and incurable brain disorder that steals its victims' memories and personality. But maze-swimming mice here are testing a remarkable vaccine that one day may reduce or prevent brain damage from Alzheimer's, which is predicted to become epidemic as the nation's elderly population grows.

Mice at the University of South Florida have been given mutated human genes that produce age-related brain degeneration much like Alzheimer's disease. To the astonishment of scientists conducting the studies, vaccinating these mice during mid-life slowed progression of their brain disorder and preserved their ability to learn.

The vaccine, developed by California scientists with Elan Corp. of Dublin, Ireland, and now undergoing safety testing in people, is one of several promising new approaches being pursued for Alzheimer's disease, a disorder whose current treatments produce only partial and short-lived improvement. In a field where progress has been glacially slow for many years, scientists now speak of intense competition and rapidly emerging discoveries.

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- Post Reporter Dr. Sus News Channel 8

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- Memory Lapse -- or A (The Washington Post, M
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Exhibit I

How healthy is your organization's technology infrastructure?

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"We tried about a dozen things that didn't work and now everything we're trying is working," said Dave Morgan, a neuroscientist at the University of South Florida who is testing the vaccine and other experimental treatments in transgenic mice. "I'm very encouraged."

The upsurge in innovative research stems in part from a clearer understanding of precisely what goes wrong in the brains of people with the disease. For example, researchers have identified two key enzymes that produce beta-amyloid, a waste protein that builds up in the brains of Alzheimer's victims and appears to be central to the destructive process. Several major drug companies are racing to identify and develop enzyme-blocking drugs called secretase inhibitors that they hope will reduce beta-amyloid accumulation. At least one company, Bristol-Myers Squibb Co., has begun testing such a drug in patients.

In addition, researchers are evaluating an array of compounds -- ranging from anti-inflammatory drugs, estrogen and cholesterol-lowering agents to various vitamins and supplements -- to see whether they can prevent Alzheimer's or delay its onset. Last month, in the first test of gene therapy for the disease, doctors in California implanted skin cells engineered to produce nerve growth factor into the brain of a woman with the disorder.

However, it may turn out that to stave off Alzheimer's disease, people will have to begin treatments such as the vaccine or enzyme-blockers in late middle age, perhaps a decade or two before symptoms would be expected to appear. At present, no medical test can predict who will develop the illness. Researchers say that if effective preventive treatments become available, such a test will be urgently needed.

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A Stealthy Assault

Like AIDS, Alzheimer's is an ultimately fatal disorder that begins its stealthy assault years before problems with memory or learning make its presence apparent.

"The baby boomers are the people now getting Alzheimer's disease," said Trey Sunderland, chief of geriatric psychiatry at the National Institute of Mental Health (NIMH). "They just don't know it."

The biggest risk factor for Alzheimer's disease is growing old. The disorder is rare in people younger than 60, but its frequency doubles every five years after 65. By age 80, about 9 percent of people have the condition; by age 90, the prevalence is 29 percent. In the next half-century, as the elderly population grows, the number of Americans with Alzheimer's disease will roughly quadruple. The total, estimated at between 2 million and 4 million now, is projected to be as high as 14 million by 2050.

The earliest sign that something is amiss in the brain is usually loss of recent memory. Later, people with the disease develop poor judgment, confusion and personality changes. They lose the ability to care for themselves and may fail to recognize their loved ones. Most live for an average of five to nine years with the illness, eventually becoming bedridden and dying of pneumonia or other infections.

Dorothy Ordway's husband and daughters first realized that she had Alzheimer's disease about six years ago, when the family rented a vacation house and Ordway kept forgetting where her bedroom was. A former banker, she was also neglecting to pay her bills.

For several years, Ordway, 80, attended a senior day care center and continued to live in her Parkville, Md., home, but she grew increasingly confused.

"She thought I was her father," recalled her husband, Thomas, 85. Last summer, after she began leaving the stove on, her family was forced to move her to an assisted-living facility.

"She knows that she's not home, but she doesn't know where she is," said her daughter, Nancy Barlow. "The day before yesterday, I'm not sure she could have told you what my name was . . . I really sensed for the first time that she wasn't quite sure."

For the family, Barlow added, watching her mother's decline "is a grieving process."

Under a microscope, the brain of someone who has died of Alzheimer's disease resembles a junkyard. Scattered among the surviving nerve cells of the cerebral cortex -- the cells responsible for thoughts, learning and decisions -- are myriad clumps or "plaques" of beta-amyloid, a waste protein toxic to nerve cells that is a hallmark of the illness. Around the plaques cluster disease-fighting cells that seem to be trying unsuccessfully to clean up or wall off the mess. Everywhere are misshapen pieces of dead nerve cells, their insides choked with tangles made of a twisted, cable-like protein called tau.

"Nerves die and all you have left are the tangles," said pathologist Juan C. Troncoso of Johns Hopkins School of Medicine as he examined such a brain. "What we're not seeing here is perhaps what is most important." The tissue specimen showed few synapses, the connections between nerve cells through which they communicate. A healthy brain cell typically has as many as 15,000 synapses with other cells. "These individuals have a tremendous amount of synaptic loss," Troncoso said.

The Vaccine Inspiration

Faced with such wreckage, researchers have tried for years to determine what sets off the destruction -- and in particular, whether amyloid plaques or tau tangles are the primary trigger. Although both appear to contribute, experts said there is now convincing evidence that buildup of beta-amyloid is at the root of the disease.

Key to this conclusion was the discovery of three human genes that, when mutated, have been found to cause inherited Alzheimer's in rare families. All three genes are involved in making beta-amyloid. One contains the code for a larger protein that is snipped apart to produce the toxic fragment; the other two carry instructions for an enzyme that does some of the snipping.

"Every known mutation ultimately increases" buildup of beta-amyloid, Morgan said.

Once these genes were identified, scientists began introducing mutated versions into the fertilized eggs of mice, hoping to engineer a mouse strain that would develop something similar to Alzheimer's disease. By the mid-1990s, scientists at Elan's laboratories in South San Francisco had such a strain and wanted to devise experiments that might lead to diagnostic tests or treatments.

At that point, biochemist Dale Schenk had an idea that he calls "a little bit crazy." Why not try vaccinating the mice against beta-amyloid?

Schenk reasoned that the protein accumulated in the brain because it was being produced faster than it was removed. He thought that if he could stimulate the immune system to make antibodies, proteins that would stick to beta-amyloid and tag it as an unwanted substance, they might shift that balance, perhaps reducing or preventing the buildup.

The idea was revolutionary because most Alzheimer's experts believe that the inflammation provoked by amyloid plaques contributes to destruction of brain cells. Many predicted that stirring up the immune system with a vaccine would only make the disease worse.

"It was breaking a lot of paradigms," Schenk acknowledged. "I had a lot of arguments with my colleagues. . . . This experiment ended up at the absolute bottom of the priority list of things to do."

Schenk first vaccinated six-week-old transgenic mice and found that the vaccine completely protected them from developing amyloid plaques. Even when the vaccine was given to older animals that already had plaques in their brains, it reduced the appearance of additional plaques and seemed to make some of the existing beta-amyloid deposits disappear. Under the microscope, it appeared that microglial cells -- wandering brain cells that clean up debris and fight infection -- were becoming activated by the vaccine and gobbling up the plaques.

"That was a major surprise," Schenk recalled.

Schenk's 1999 paper on the Elan vaccine created a sensation, not least because the unexpected findings suggested that vaccines might be helpful in disorders where no one had thought of using them. His results have since been confirmed by other researchers.

But no one knew whether the treatment could improve learning or memory in affected animals. Without such evidence, medical researchers would be reluctant to try it in people. "You may remove the amyloid, but patients may not do any better," noted Hopkins' Troncoso.

Using the Florida transgenic mice, Morgan and his team tried to address that question. They used a water maze shaped like a daisy with six petals to test animals' "working memory": the ability to learn and remember new information, which is the earliest brain function affected by Alzheimer's.

Each day, the escape platform is placed at the end of a different arm of the maze. A mouse must swim until it locates the platform, which is invisible from the surface. Mice in the experiment were given five trials each day, testing their ability to learn and recall the platform's location. The next day, the platform was moved to a new location.

"It's like you have to remember where you parked your car," said David Diamond, a behavioral neuroscientist who designed the water maze used in the study.

Morgan and his colleagues gave transgenic animals monthly injections of a vaccine similar to the one developed by Elan, starting at seven months of age. He first tested them in the maze when they were 11 months old, expecting that brain inflammation caused by the vaccine would worsen their performance. Instead, they learned the maze as fast as normal mice. "We were completely wrong," he said. "They were just dynamite."

By 15 months of age, transgenic mice that had not gotten the vaccine had developed severe brain disease and could no longer navigate the maze. But the vaccine recipients could still learn and remember the platform's location, although they took longer to master it than normal animals. Morgan and his team are now studying whether the vaccine still protects the brain when the treatment is begun later in the animals' lives.

Testing in Humans

The Florida team's promising findings and those of another group in Canada have spurred Elan's efforts to test the vaccine in Alzheimer's patients. Last year, a small safety study in this country found no significant side effects. The vaccine is now undergoing a multidose safety trial involving about 80 patients in Great Britain who have mild or moderate Alzheimer's disease. The results are expected within the next two months. If they are favorable, the company hopes to begin testing the vaccine in a larger number of patients to see whether it has a favorable impact on their illness.

"I think it provides some hope," said Schenk, who is to receive a prize for his discovery today at the American Academy of Neurology's annual meeting.

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Many researchers are nervous about the prospect of giving a vaccine to activate the immune system in the brain, reasoning that if it triggers inflammation or other adverse effects, doctors won't be able to turn off the process. Some have suggested it might be safer simply to give patients periodic injections of antibodies against beta-amyloid -- much as gamma globulin shots were once given to prevent hepatitis -- because the treatment could be stopped if side effects developed.

The NIMH's Sunderland, who is trying to develop a predictive test for Alzheimer's disease, is studying a group of healthy volunteers who are at higher-than-average risk because they have parents or siblings with the disorder.

"They ask, 'Should I get the vaccine?'" Sunderland said. "My opinion is, 'No. Not now.' "

Nevertheless, Sunderland said he is encouraged by the results so far. He said he suspects that an Alzheimer's vaccine may work better for preventing the disease than for treating it once the brain has become severely affected.

"Let's say they give it to Alzheimer's patients and it fails," he said. "It might seem a devastating blow to the vaccine concept, but maybe they gave it to the wrong people. Right now, there is no flashlight . . . telling you where to point your treatment, and when."

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PTO/SB/22 (12-04)

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) **FY 2007** ***(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)***	Docket Number (Optional) 15270J-004747US
Application Number 10/828,548	Filed April 19, 2004
For PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE	
Art Unit 1649	Examiner Kolker, Daniel E.

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$120	$60	$
☐ Two months (37 CFR 1.17(a)(2))	$450	$225	$
☒ Three months (37 CFR 1.17(a)(3))	$1020	$510	$ 1020.00
☐ Four months (37 CFR 1.17(a)(4))	$1590	$795	$
☐ Five months (37 CFR 1.17(a)(5))	$2160	$1080	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 19-4880.

WARNING: Information on this form may become public. Credit card information should not be included on this form.
Provide credit card information and authorization on PTO-2038.

I am the
☐ applicant/inventor.
☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).
☐ attorney or agent of record. Registration Number
☒ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 42,397

Rosemarie L. Celli
Signature

April 24, 2007
Date

Rosemarie L. Celli, Reg. No. 42,397
Typed or printed name

650.625.8100
Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☒ Total of 1 forms are submitted.

57583

PATENT ABSTRACTS OF JAPAN

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(21)Application number : 61-109433

(22)Date of filing : 15.05.1986

(71)Applicant : TOKYO MET GOV SEISHIN IGAKU SOGO KENKYUSHO

(72)Inventor : ISHII TAKESHI
SHINODA TOMOTAKA

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(54) MONOCLONAL ANTIBODY REACTIVE TO SENILE SPOT, CELL STRAIN PRODUCING SAME AND PRODUCTION OF SAID MONOCLONAL ANTIBODY

(57)Abstract:

NEW MATERIAL:A monoclonal antibody reactive to senile spot in cerebral structure having 160,000W180,000mol.wt. as a monomer and 800,000W1,000,000mol. wt. as pentamer by polyacrylamide gel secondary electrophoresis using sodium dodecyl sulfate as a protein modifier, 6.3W8.3 isoelectric point, 0.48W0.62 mobility of monomer and 0.11W0.17 mobility of pentamer.

USE: A diagnosticum for Alzheimer's senile dementia and Alzheimer's disease.

PREPARATION: For example, a splenic cell which is obtained by immunizing a mouse against amyloid protein separated from autopsy spleen of a patient of human protopathic amyloidosis as an antigen and a mouse myeloma cell are subjected to cell fusion, the formed fused cell is cloned by limiting dilution method into a monoclone and then the monoclonal cell is cultivated to obtain a monoclonal antibody from the supernatant liquid of the culture mixture.

LEGAL STATUS

[Date of request for examination]

⑲日本国特許庁（JP）　　⑪特許出願公開

⑫公開特許公報（A）　　昭62-267297

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//(C 12 P 21/00		
C 12 R 1:91)		

㊸公開　昭和62年(1987)11月19日

審査請求　未請求　発明の数　3　（全13頁）

㊹発明の名称　老人斑反応性モノクローナル抗体、それを産生する細胞株及び該モノクローナル抗体の製造方法

㉑特　願　昭61-109433

㉒出　願　昭61(1986)5月15日

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⑭代理人　弁理士　阿形　明

明　細　書

1．発明の名称　老人斑反応性モノクローナル抗体、それを産生する細胞株及び該モノクローナル抗体の製造方法

2．特許請求の範囲

1　タンパク変性剤としてドデシル硫酸ナトリウムを用いたポリアクリルアミドゲル二次元電気泳動によって、単量体としての分子量160,000～180,000及び5量体としての分子量800,000～1,000,000を示すとともに、タンパク変性剤を用いないポリアクリルアミドゲル二次元電気泳動における等電点が6.3～8.3の範囲にあり、かつ単量体としての移動度が0.48～0.62及び5量体としての移動度が0.11～0.17の範囲にある老人斑反応性モノクローナル抗体。

2　イムノグロブリンMクラスに属する特許請求の範囲第1項記載のモノクローナル抗体。

3　タンパク変性剤としてドデシル硫酸ナトリウムを用いたポリアクリルアミドゲル二次元電気泳動によって、単量体としての分子量160,000～180,000及び5量体としての分子量800,000～1,000,000を示すとともに、タンパク変性剤を用いないポリアクリルアミドゲル二次元電気泳動における等電点が6.3～8.3の範囲にあり、かつ単量体としての移動度が0.48～0.62及び5量体としての移動度が0.11～0.17の範囲にある老人斑反応性モノクローナル抗体を産生する細胞株。

4　モノクローナル抗体がイムノグロブリンMクラスに属するものである特許請求の範囲第3項記載の細胞株。

5　マウス骨髄腫細胞とアミロイドタンパクを抗原としてマウスに免疫して得られた脾細胞とを細胞融合させて成る細胞株にモノクローナル抗体を産生させることを特徴とする、タンパク変性剤としてドデシル硫酸ナトリウムを用いたポリアクリルアミドゲル二次元電気泳動によって、単量体としての分子量160,000～180,000及び5量体としての分子量800,000～1,000,000を示すとともに、タンパク変性剤を用いないポリアクリルアミドゲル

二次元電気泳動における等電点が6.3～8.3の範囲にあり、かつ単量体としての移動度が0.48～0.62及び５量体としての移動度が0.11～0.17の範囲にある老人斑反応性モノクローナル抗体の製造方法。

6　アミロイドタンパクが、ヒト原発性アミロイドーシス患者に沈着したアミロイドタンパクをアルカリ処理して変性したものである特許請求の範囲第５項記載の方法。

7　モノクローナル抗体がイムノグロブリンＭクラスに属するものである特許請求の範囲第５項記載の方法。

3．発明の詳細な説明

産業上の利用分野

本発明は、老人斑に特異的に反応するモノクローナル抗体、それを産生する細胞株及び該モノクローナル抗体の製造方法に関するものである。さらに詳しくいえば、本発明は、老年痴呆症の診断に有用な、脳組織における老人斑構成物質及びそのタンパク質と相同性の高い脳血管沈着物質と特異的に反応するモノクローナル抗体、それを産生する細胞株及び該モノクローナル抗体の製造方法に関するものである。

従来の技術

近年、人口構成が高齢化するに伴い、老年痴呆症が社会問題となりつつある。この老年痴呆症の中でもアルツハイマー型老年痴呆及びアルツハイマー病(以下、この両者を合わせてSDATと略す)に関しては、病因が不明であって、治療法のみならず明確な診断方法も確立されていないのが現状である。

現在、SDATの診断方法としては、主として患者の言動から痴呆の程度を求める臨床知見によるものと、脳の剖検、生検によって得た病理知見によるものとがあり、診断確立は患者の死後に脳の剖検によることが多い。

SDATの病理知見からの診断指標の１つとして、患者の脳に正常老人よりもはるかに多く沈着する老人斑の数が採用されている。すなわち、剖検又は生検によって得たSDAT患者脳切片をコンゴーレッドを用いて染色し、光学通常顕微鏡下で赤く染まり、かつ偏光顕微鏡下で緑色の複屈折を示す顆粒状のもので、斑状に分布する老人斑を計数し、その数の多いことをSDATの診断の１つの根拠としている。これは、老人斑を構成するアミロイドタンパクのアミロイドとしての性質に基づくものである〔「臨床神経学」第２２巻、第1106～1108ページ(1982年)、「日本老年医学会雑誌」第１９巻、第354～358ページ(1982年)、「神経内科」第１２巻、第235～243ページ(1980年)〕。

しかしながら、このようなコンゴーレッド染色による老人斑の確認方法は、アミロイドタンパクに共通の確認方法であり、老人斑に特異的でなく、また、偏光下での緑色複屈折の色調も微妙で、偏光下で類似の色調を示す他の物質との識別が容易でないことが多い上に、アミロイドタンパクがある程度の大きさ以上の顆粒状などの構造体にならなければ検出できず、検出感度が低いなどの欠点を有している。

また、この老人斑が過ヨウ素酸－シッフ(PAS)染色陽性であることを利用して、脳切片中の老人斑を検索し、SDATの診断指標の１つとすることも行われているが〔「神経内科」第１２巻、第235～243ページ(1980年)〕、この過ヨウ素酸－シッフ(PAS)染色法は、主として糖類の組織化学的染色に用いられるものであり、老人斑のみに特異的に反応するものではない。

さらに、該老人斑と反応する抗体としては、例えば抗ヒト免疫グロブリン抗体〔「アクタ・ニューロパソロジカ(acta neuropathol.)」第３２巻、第157～162ページ(1975年)、同第３６巻、第243～249ページ(1976年)など〕、抗ヒトプレアルブミン抗体〔「アメリカン・ジャーナル・オブ・パソロジー(American Journal of Pathology)」第107巻、第４１～５０ページ(1982年)〕、抗ヒト補体抗体〔「アクタ・ニューロパソロジカ(acta neuropathol.)」第６３巻、第296～300ページ(1984年)、同第５７巻、第239～242ページ(1982年)〕などが報告されている。しかしながら、これらの抗体はいずれもポリクローナル抗体であり、しかもその目的は老人斑の検索ではなく、老人斑構成

又は随伴タンパクの性質を明らかにしようとしたものであるし、またこれらの抗体を老人斑の検索に用いたとしても、該抗体は老人斑に対して特異的なものでないため、脳内の老人斑以外に存在する免疫グロブリン、プレアルブミン、補体とも反応するという問題がある。

従来、老人斑アミロイドタンパクをそのまま抗原として動物に免疫し、抗血清を得る方法も試みられているが、アミロイドの難溶性のため、力価の高い抗体は得られていない。また、この方法により得られる抗血清やポリクローナル抗体は、特異性、生産性及び品質の安定性に問題がある。

例えば、抗血清をイオン交換クロマトグラフィーなどにより免疫グロブリン分画を回収して得られるポリクローナル抗体は、特異性の低い抗体や、免疫に使用した異物中に混在する所望のものとは異なる異物に対して反応する抗体も含んでおり、その特異性において不十分であり、実用には適さない。また、動物を免疫するために絶えず抗原となる特定のタンパク質が必要であり、かつこのタンパク質の品質が変われば、当然免疫された動物から得られる抗体も品質が変わる上に、動物の個体間でも得られる抗体の力価が異なるので、安定した品質の抗体を得ることは困難である。さらに、動物を免疫してから、その抗血清を得るまでには、通常１～３か月を要し、その間免疫強化注射や動物の飼育などに多くの労力が必要となるので実用的でない。

ところで、モノクローナル抗体は、抗血清より得られる抗体が種々の抗体の混合物であるのに対して、ただ１種類の抗体(すなわち、モロクローナル抗体)のみから成るため、常に一定の抗原特異性を示す。このモノクローナル抗体は、細胞融合法〔「ネイチャー(Nature)」第256巻、第495～497ページ(1975年)〕によって、抗体産生株を新たに形成せしめ、この抗体産生株より得られることが知られている。また、ある種のウィルス(Epstein-Barr Virus)などを用いて、抗体産生能を有する正常細胞を長期培養可能な抗体産生株に変異させて、その抗体産生株よりモノクローナル抗体を得ることも可能である。

前者の細胞融合法について、さらに詳しく説明するならば、例えば、マウスなどの免疫可能な動物を抗原で免疫し、免疫成立後、その動物から脾臓などを外科的に取り出すことなどによって、抗体産生能を有する細胞を入手する。この抗体産生能を有する細胞(リンパ球Ｂ細胞)と、ある種のマーカーを持つ無限増殖性細胞株(以下、単に親株と称す)とを融合促進剤の存在下、あるいはある種のウィルスの存在下で融合する。ここで用いる親株のマーカーとしては、一般にある種の成分を欠いた培養液中、あるいはある種の成分を含む培養液中で生存できないことがよく利用される。例えば、DNA合成回路(サルベージ回路)においてDNA合成に関与する酵素であるヒポキサンチン・グアニン・ホスホリボシル・トランスフェラーゼ(Hypoxanthine-Guanine Phosphoribosyl Transferase：HGPRT)あるいはチミジンキナーゼ(Thymidine Kinase：TK)を欠損させたものが利用される。すなわち、HGPRTやTKの酵素をもつ細胞では、DNA合成回路(de novo回路)におけるDNA合成阻害物質であるアミノプテリンを含む培養液〔ヒポキサンチン・アミノプテリン・チミジン(Hypoxanthine Aminopterin Thymidine：HAT)を含む選別用培養液(HAT培地)〕で培養すると、アミノプテリンによってDNA合成回路(de novo回路)が阻害されても、HGPRTあるいはTKなどの酵素によって、レスキュー回路(rescue pathway)であるサルベージ回路が働き、DNA合成が行われるのに対して、HGPRTあるいはTKのような酵素を欠損した細胞では、HGPRTあるいはTKなどの酵素によるサルベージ回路が働かないため、アミノプテリンによってDNA合成回路(de novo回路)が阻害されると、DNA合成は不可能となり、HAT培地中では生存できないことになる。

このようにして親株と正常細胞である抗体産生能を有する細胞との融合後、親株と融合細胞とを、親株が持つマーカーによって分離し、融合細胞のみを選択することができる(融合しなかった抗体産生能を有する細胞は正常細胞であるため、培養

を続けることによって死滅してしまう）。このようにして得られた融合細胞より、目的とする抗体を産生するただ１個の細胞より分裂増殖した細胞群を選択し、この細胞群よりモノクローナル抗体を産生させることができる。

一方、老人斑のアミロイドタンパク質の性質については、アミノ酸組成〔「アーク・ニューロロジィ(Arch. Neurol.)」第２５巻、第198～211ページ(1971年)、「ブレイン・リサーチ(Brain Research)」第２４巻、第259号、第348～352ページ(1983年)など〕、及びアミノ酸配列の一部〔「プロシーディングズ・オブ・ナショナル・アカデミィー・オブ・サイエンス USA(Proc. Natl. Acad. Sci. USA)」第82巻、第4245～4249ページ(1985年)〕が報告されている。またSDAT患者の脳血管に沈着するアミロイドのアミノ酸配列も報告されており〔「バイオケミカル・アンド・バイオフィジカル・リサーチ・コミュニケイション(Biochemical & Bio-physical Research Communication)」第120巻、第885～890ページ(1984年)〕、このものは、老人斑アミロイドのアミノ酸配列と高い相同性があることも知られている。

しかしながら、これらの知見を利用して、実用的な老人斑反応性モノクローナル抗体を産生する技術はまだ確立されていない。

発明が解決しようとする問題点

本発明は、このような事情のもとで、SDAT患者の脳組織における老人斑構成物質及びそのタンパク質と相同性の高い脳血管沈着物質と特異的に反応するモノクローナル抗体を産生する細胞株を確立し、この細胞株より産生された該老人斑反応性モノクローナル抗体を提供することを目的としてなされたものである。

問題点を解決するための手段

本発明者らは前記目的を達成するために鋭意研究を重ねた結果、意外にもアミロイドタンパク、特に原発性アミロイドーシス患者の剖検脾から抽出したアミロイドタンパクのアルカリ処理物を抗原としてマウスに免疫して得られる脾細胞とマウスの骨髄腫細胞とを細胞融合することにより、目的とするモノクローナル抗体を産生する細胞株が得られることを見出し、この知見に基づいて本発明を完成するに至った。

すなわち、本発明は、タンパク変性剤としてドデシル硫酸ナトリウムを用いたポリアクリルアミドゲル二次元電気泳動によって、単量体としての分子量160,000～180,000及び５量体としての分子量800,000～1,000,000を示すとともに、タンパク変性剤を用いないポリアクリルアミドゲル二次元電気泳動における等電点が6.3～8.3の範囲にあり、かつ単量体としての移動度が0.48～0.62及び５量体としての移動度が0.11～0.17の範囲にある老人斑特異的モノクローナル抗体及びそれを産生する細胞株を提供するものである。該モノクローナル抗体は、マウス骨髄腫細胞とアミロイドタンパクを抗原としてマウスに免疫して得られる脾細胞とを細胞融合させて成る細胞株にモノクローナル抗体を産生させることによって、製造することができる。

本発明において用いる抗原タンパクは、アミロイドタンパク、好ましくはヒト原発性アミロイドーシス患者に沈着したアミロイドタンパク(通常ALタンパクと呼ばれる)、特に好ましくは、このALタンパクをアルカリ処理して成る変性アミロイドタンパク(以下変性ALタンパクとする)である。この変性ALタンパクの好適な製造方法の１例を示すと、ヒト原発性アミロイドーシス患者の脾臓、肝臓、腎臓などの臓器や関節などにはアミロイドタンパクが沈着しているので、まず、アミロイドタンパクを含有する前記臓器をホモジナイズしたのち、このホモジネートから非アミロイドタンパクを0.1～0.2M程度の濃度の食塩水で抽出除去し、残の粗アミロイドタンパクを水抽出により溶液状態とし、次いで、この溶液に塩濃度が0.1～0.2M程度になるように塩化ナトリウムなどを加えて、粗アミロイドタンパクを沈殿させたのち、この沈殿を0.05～0.15M程度の濃度の水酸化ナトリウム水溶液により室温で１０～３０時間処理後、中和することによって該変性ALタンパクが得られる。

抗原タンパクとしては、SDAT患者の脳から単離

した老人斑アミロイドタンパクを用いることも可能であるが、このものは溶解度が低く、かつ抗原とするのに十分な量を得ることが容易でない上、抗原性が低いなどの問題があるので、抗原タンパクとしては、前記のALタンパク、特に変性ALタンパクが好適である。

本発明においては、前記抗原タンパクを通常の方法によりマウスに免疫したのち、その脾臓を取り出し、細胞融合の一方の細胞とする。例えば、抗原アミロイドタンパクをフロイントの完全アジュバントなどと共に、BALB/Cマウスに免疫し、免疫成立後、そのマウスより脾臓を外科的に取り出すことによって抗体産生能を有する細胞が得られる。

次に、このようにして得られたマウス脾細胞とマウス骨髄腫細胞(親株)とを好ましくは融合促進剤の存在下で細胞融合する。この親株としては種々の株が報告されており、前記マウス脾細胞に適した親株が選ばれ、該脾細胞と細胞融合される。前記BALB/Cマウス由来の脾細胞と細胞融合させる親株としては、例えばBALB/Cマウスのミエローマ細胞由来のHGPRT欠損細胞株であるP3-X63-Ag8株などが用いられる。

細胞融合の際に用いられる融合促進剤としては、各種分子量のポリエチレングリコール(PEG)が一般によく用いられるが、人工脂質小胞であるリポソーム(liposome)やセンダウィルス(HVJ)なども用いることができる。また、これらの融合促進剤を用いずに、細胞に電圧をかけることによって細胞融合する電気融合法も知られている。

親株としてP3-X63-Ag8株を用い、細胞融合した場合、融合後に、HAT培地で培養することによって、抗体産生能を有する細胞(正常細胞)とP3-X63-Ag8とから成る融合細胞のみを選択することができる。

このようにして得られた融合細胞の中で、本発明に係る抗原と最もよく反応する抗体を産生する融合細胞(抗体産生株)は、融合細胞の培養上清を用いて、特異抗体測定のための免疫学的測定法によって選択できる。

この特異抗体の測定は、例えばポリスチレン製マイクロプレートなどを固相として、特異抗原である変性ALタンパク質を吸着させ、次に融合細胞培養上清を加えて反応させる免疫測定法によって行うことができる。例を挙げて詳しく述べるならば、まずポリスチレン製マイクロプレートに該タンパク質を吸着させる。この際、タンパク質吸着用緩衝液としては、一般に炭酸ナトリウム・炭酸水素ナトリウム緩衝液が好ましく用いられている。あるいはリン酸緩衝液などを用いることも可能である。本発明者らの経験では、吸着の際の特異抗原又は対照抗原の濃度は１～１０μg/mℓで十分であるが、この濃度未満でも、抗体濃度や以下の反応条件を変えることによって、十分に測定できた。この炭酸ナトリウム・炭酸水素ナトリウム緩衝液などで至適濃度に調製された特異抗原又は対照抗原を、ポリスチレン製マイクロプレートへ一定量ずつ加え、一定時間静置する。これは、４℃で一晩放置するのが最も一般的であるが、その他、室温で２時間程度静置することも可能である。あるいは３７℃で１時間静置によっても可能である。このようにして抗原を感作したポリスチレン製マイクロプレートを、例えば界面活性剤を含むリン酸緩衝液などによって洗浄したのち、融合細胞培養液中の抗体を一定時間反応させる。上記と同様にしてポリスチレン製マイクロプレートを洗浄したのち、あらかじめ決定しておいた希釈倍率に希釈した酵素標識抗マウスイムノグロブリン(Ig)抗体を加えて、ポリスチレン製マイクロプレート上で抗原・抗体反応した抗体と反応させる。さらに上記と同様にしてポリスチレン製マイクロプレートを洗浄したのち、酵素基質を加えて酵素活性を測定する。ここで測定できた酵素活性は、ポリスチレン製マイクロプレートに吸着した抗原と反応した、融合細胞培養液中の抗体の量を間接的に示している。これによって、融合細胞培養液中の抗体の特異性を測定できる。また、ここでは酵素標識抗マウス抗体を用いた酵素免疫測定法について述べたが、この他、ラジオアイソトープで標識した抗マウスIg抗体を用いて、同様の手段で行うことも可能である。

その他、一般に用いられる抗体の特異的検出法

によってもできる。

これらのスクリーニング法と、例えば限界希釈法やソフトアガーを用いる方法などによるクローニング法との組合せによって、最終的に目的とする抗体を産生する単一の細胞クローンである抗体産生株を含む一群のクローンを確立できる。

このようにして得られたクローンから、モノクローナル抗体を得、これを用いてアルツハイマー型老年痴呆(SDAT)患者脳を免疫組織化学的に検索し、老人斑と強く反応する抗体及びその産生株を選択する。このようにして、目的とするモノクローナル抗体及びその産生株を得ることができる。

抗体産生株からモノクローナル抗体を得るには、例えばまずモノクローナル抗体産生株を、プリスタンなどであらかじめ刺激したマウスの腹腔に注入し、一定期間経過後、その動物の腹腔にたまった腹水を採取するか、あるいは抗体産生株を培養し、培養上清を採取する。このようにして採取したモノクローナル抗体を含む液から、通常行われている抗体の精製方法に従って、目的とするモノクローナル抗体を得ることができる。

この抗体を精製するには、例えば腹水に硫酸ナトリウムなど通常塩析に用いられる塩を加えて塩析し、得られた沈殿を遠心分離によって回収したのち、この沈殿を、リン酸緩衝液などのような中性の緩衝液で溶解し、次いで透析などによって、硫酸ナトリウムなど塩析に用いた塩を除去する。これから、イオン交換クロマトグラフィーなどの通常行われている抗体の精製方法によって、目的とするモノクローナル抗体を含む分画を回収することができる。そのほか、細胞株の培養上清を濃縮したのち、前記精製方法を行うか、又は抗マウスIg抗体、抗原に用いた変性ALタンパク、プロテインＡなどを用いたアフィニティクロマトグラフィ法によって、目的とするモノクローナル抗体を回収することもできる。

このようにして得られた本発明のモノクローナル抗体は、イムノグロブリンＭ(IgM)又はイムノグロブリンＧ(IgG)クラスであることが多く、特にIgMクラスである場合が多い。また、タンパク変性剤としてドデシル硫酸ナトリウムを用いたポリアクリルアミドゲル二次元電気泳動によって、単量体としての分子量160,000~180,000、及び5量体としての分子量800,000~1,000,000を示し、タンパク変性剤を用いないポリアクリルアミド二次元電気泳動における等電点が、タンパクの泳動位置に相当するpHをそのタンパクの等電点として6.3~8.3の範囲を示す。さらに、タンパク変性剤を用いないポリアクリルアミドゲル二次元電気泳動における移動度が、アルブミンの最先端部の移動度を1.0としたとき、単量体として0.48~0.62、5量体として0.11~0.17の範囲を示す。このように、本発明のモノクローナル抗体は主としてIgMクラスであるので、単量体(分子量約170,000前後)と5量体(分子量約900,000前後)の混合物として得られることが多い。

本発明のモノクローナル抗体を用いた脳切片の免疫組織化学的検索は、通常の方法によって行われる。すなわち、死後凍結脳又はホルマリンなどで固定したパラフィン封入脳などから組織切片を作成し、これをトリプシンで短時間処理する。この処理は、通常0.1%トリプシンを用い、３７℃の温度で１０分間行われる。このようなトリプシン処理は必ずしも必要ではないが、トリプシン処理した方が老人斑と本発明のモノクローナル抗体との反応が強くなり、非特異的な反応も抑制することができる。

このようにして得た切片を、本発明のモノクローナル抗体を用いて、パーオキシダーゼーアンチパーオキシダーゼ(PAP)法やアビジンービオチン(ABC)法などにより免疫染色する。パーオキシダーゼによる発色の基質としてはジアミノベンジジンなどが一般に用いられる。

また、本発明のモノクローナル抗体をローダミン、フルオレセインイソチオシアネート(FITC)などの色素で標識することにより、酵素を介さず直接老人斑を染めることもできる。さらに、放射性同位元素で本発明のモノクローナル抗体を標識し老人斑と反応させることもできる。この場合は、老人斑の量が放射能でカウントできるので迅速か

つ容易な老人斑の定量方法ともなる。

このような本発明のモノクローナル抗体を用いる方法を、従来のコンゴーレッドによる染色後、偏光下で緑色の複屈折をみる方法や電子顕微鏡によるアミロイド繊維の確認方法などと組み合わせることにより、本発明のモノクローナル抗体が老人斑のアミロイド繊維と特異的に反応することが明らかとなった(実施例参照)。

なお、本発明のモノクローナル抗体は、一部SDAT患者の脳血管に沈着するアミロイドと弱く反応することがあるが、これは老人斑アミロイドタンパクと脳血管アミロイドタンパクとの相同性により、同じ抗原を認識するものと思われる。また、本発明のモノクローナル抗体は、臓器切片上において抗原としたALタンパクから成るアミロイドとは反応しない。

本発明のモノクローナル抗体が認識する抗原物質は明確ではないが、アミロイド繊維そのもの又は付随するタンパクや糖タンパクなどであると考えられる。

発明の効果

本発明によると、SDAT患者の脳に多数みられる老人斑タンパク質など及びそのタンパク質と相同性の高い脳血管に沈着するタンパク質などに対して特異的に反応するモノクローナル抗体を産生する細胞株が提供され、老人斑特異的なモノクローナル抗体を得ることが可能になったため、免疫学的手法を用いた脳の老人斑の高感度かつ高特異性の検索を行うことができる。

また、本発明のモノクローナル抗体は、SDAT患者の他の脳の組織、例えばミエリン(myelin)、軸索〔アクソン(axon)〕、神経細胞〔ニューロン(neuron)〕、グリア(glia)細胞とは免疫組織化学的に反応せず、したがって、該モノクローナル抗体を用いることにより、老人斑の検索が極めて容易に行える。

さらに、本発明のモノクローナル抗体を用いて、血清又は脳脊髄液中に存在すると思われる老人斑に特異的な構成タンパク質又はその前駆タンパクなどを検索することが可能となり、SDATの早期診断にも有用であると思われる。

また、本発明の抗体産生株は、常に一定の抗原特異性や抗原との結合力を有する抗体(すなわち、モノクローナル抗体)を産生し、かつ、動物を免疫して得られる抗体が多種多様な抗体の混合物であるのに対して、単一の抗体であるため、一定の力価に調整することが容易である。つまり、安定した品質の抗体を安定供給できるなど工業的にも有用である。

実施例

次に実施例により本発明をさらに詳細に説明する。

実施例1　モノクローナル抗体産生株の調製

(1)　抗原の調製

原発性アミロイドーシス患者の剖検脾10gを氷冷した0.15M塩化ナトリウム－0.05%アジ化ナトリウム溶液100mℓ中に入れ、ホモジナイザーにより約3000rpmの回転数で5分間処理して脾のホモジネートを得た。このホモジネートを4℃で、12000×gで30分間遠心分離し、上清の280nmにおける吸光度(OD_{280})を測定した。沈渣を前記0.15MNaCℓ－0.05%NaN_3溶液100mℓに再度懸濁し、前記の遠心分離を行うという操作を、OD_{280}が0.05以下となるまで5回(計6回)繰り返した。

このようにして得られた沈渣に氷冷した蒸留水80mℓを加え、5分間約3000rpmでホモジナイズしたのち、このホモジネートを4℃、12000×gで30分間遠心し、上清を得た。また、この際得られた沈殿については、前記の氷冷蒸留水処理をさらに行い、遠心処理後の上清(2回目の上清)を得、2回目の氷冷蒸留水処理の沈渣を再度氷冷蒸留水処理して3回目の上清を得た。1回目、2回目、3回目の上清を混合して粗アミロイドタンパク溶液を得た。

次に、このようにして得られた粗アミロイドタンパク溶液に4℃の条件下、塩化ナトリウムを加えその濃度が0.15Mとなるようにした。この操作により、析出、沈殿してくる粗アミロイドタンパクを、4℃、12000×gで60分間遠心分離することによって集めた。次いで得られた沈渣10.5mgを

0.1M水酸化ナトリウム水溶液で室温中１６時間処理することにより可溶化した。この0.1MNaOH溶液を0.1M塩酸で中和して抗原溶液を得た。

（２） マウスへの免疫

（１）で得た抗原溶液(タンパク濃度500μg／mℓ)0.1mℓに等量のフロイント完全アジュバント(Freund's complete adjuvant)を加えて十分に混和した。この完全な油中水型エマルジョンとしたものを雌の７週令のBALB/Cマウスに皮下注射した。さらに１か月半後に同じ抗原溶液0.1mℓを腹腔に注入して免疫強化(boost)した。免疫強化の３日後に脾臓を取り出し、ダルベッコの最少基本培地(Minimum Essential Medium、以下DMEM培地と略す)を注射器で脾臓に注入し、脾細胞を洗い出し分散させ、さらにメッシュを通すことにより脾膜を取り除いた。

（３） 細胞融合

マウスのミエローマ細胞株P3X63-Ag8の細胞２×１０^{7}個と、（２）で得た脾臓細胞8.6×１０^{7}個とをDMEM培地(無血清)中で十分に混合したのち、

遠心分離して上清を捨てた。この沈渣にDMEM培地2.0mℓ当り、ポリエチレングリコール4000(PEG4000)2.0gを溶解した液1.0mℓを室温で１分間要して加えたのち、３７℃の湯浴中で９０秒間インキュベートして融合を行わせた。次いでDMEM培地９mℓを室温で徐々に加え、さらに５分経過後DMEM培地10mℓを添加した。

これらの細胞を十分に洗浄したのち、ヒポキサンチン１×１０$^{-4}$M、アミノプテリン４×１０$^{-7}$M、チミジン1.6×１０$^{-5}$M、ウシ胎児血清１０％を含むMEM培地(以下HAT培地という)を用い、９６穴培養プレート中で培養した。HAT培地は３日おきに交換し、細胞融合２週間後に、アミノプテリンを含まない以外は、前記HAT培地と同じ培地(これをHT培地と略す)に切り換え、コロニー状に生育してくる融合細胞を選択した。

（４） 酵素免疫測定法による融合細胞の選別（３）で得た融合細胞の産生する抗体の力価の測定を培養開始２週間後に以下のようにして行い、免疫に用いた抗原タンパクと反応する抗体を産生する雑

種細胞株を選別した。

ⅰ） マイクロプレートの抗原感作

免疫に用いたヒト原発性アミロイドーシス患者脾アミロイドタンパクの水酸化ナトリウム処理物〔特異抗原〕を0.02M炭酸ナトリウム・炭酸水素ナトリウム緩衝液(pH9.6)でタンパク濃度１０μg／mℓとなるように調製した。この液をポリスチレン製マイクロプレートの各ウェルに100μℓずつ加えて、蒸発を防いで４℃で一晩静置し、タンパク質を吸着させた。

ⅱ） 一次反応

こうして物理吸着によって特異抗原を固定したポリスチレン製マイクロプレート(以下、単にマイクロプレートと称す)を、リン酸緩衝生理食塩液(塩化ナトリウム8.0g／ℓ、リン酸一カリウム0.2g／ℓ、リン酸二ナトリウム・７水塩2.17g／ℓ、塩化カリウム0.2g／ℓ、ツィーン２０(Tween２０)0.5mℓ／ℓ、アジ化ナトリウム0.2g／ℓを含む液、pH7.4、以下、PBSTと称す)で洗浄した。

次に、培養上清の非特異的吸着を防ぐ目的で、このマイクロプレートに１０％正常ウマ血清(200μℓ／ウェル)を加え室温で１時間ブロッキングを行った。

次いで、ウマ血清を除いたのち、融合細胞の培養上清(100μℓ／ウェル)を加えて反応させた〔室温(２２～２５℃であった)、２時間〕。この際、培養上清中に、マイクロプレートに固定した特異抗原との反応性を有する抗体が存在すれば、その抗体は、抗原・抗体反応によってマイクロプレート上に保持される。

ⅲ） 二次反応

反応後、PBSTによってマイクロプレートを洗浄した。次に、あらかじめ決定した至適希釈倍数にPBSTによって希釈したアルカリフォスファターゼ結合抗マウス〔IgG＋IgM〕抗体液を、マイクロプレートの各ウェルに100μℓずつ添加して、室温(２２～２５℃)で２時間反応させた。このアルカリフォスファターゼ結合抗マウス〔IgG＋IgM〕抗体は、マイクロプレート上に

保持された培養上清中のマウスIgG及びIgMと反応する。

iv）　酵素活性の測定

二次反応後、PBSTでマイクロプレートを洗浄したのち、マイクロプレート上に保持されたアルカリフォスファターゼ活性を測定した。酵素基質であるパラニトロフェニルリン酸を、ジエタノールアミン緩衝液〔ジエタノールアミン97mℓ/ℓ、塩化マグネシウム・6水塩100mg/ℓ、アジ化ナトリウム0.2g/ℓを含む液を塩酸を用いてpH9.8に調整した液〕にて1mg/mℓとなるように溶解した液を酵素基質溶液とした。この酵素基質溶液をマイクロプレートの各ウェルに100μℓずつ添加して、室温(２２～２５℃)で１時間反応させた。反応後、１Ｎ水酸化ナトリウム液を５０μℓずつ各ウェルに加えて酵素反応を止めた。各ウェルの酵素基質溶液の波長405nmにおける吸光度を測定して、酵素活性を測定した。この酵素活性は、マイクロプレート上の特異抗原あるいは対照抗原と反応した、培養上清中の抗体量を間接的に示している。

以上の測定法と、限界希釈法によるクローニングとを３回繰り返して、特異抗原と反応する抗体を産生する、単一の細胞由来の細胞集団(すなわち、モノクローナルな新規雑種細胞)を６０クローン得た。

実施例２　老人斑に対する反応性と特異性の確認

実施例１(４)で得た６０クローンの産生するモノクローナル抗体を用いて老人斑に対する反応性を検討した。

すなわち、アルツハイマー型老年痴呆患者の死後剖検脳３例の大脳皮質から厚さ２mmの切片を切りとり、ティッシューーテックO.C.T.コンパウンド(Tissue-Tek O.C.T. Compound、ラブーテックプロダクト社製)中に包埋し、液体窒素でただちに凍結した。これより、厚さ１０μmのクリオスタット切片を作成し、スライドグラス上にマウント(mount)し、風乾後１０分間アセトンで固定した。

このスライドグラス上の切片を0.1%トリプシン溶液で３７℃、１０分間処理したのち、トリス緩衝食塩水(Tris buffer saline、以下TBSと略す)で３回洗浄した。これをTBSで６倍に希釈した馬血清で２０分間処理し、非特異的吸着を防止した。

このように処理された脳切片を、実施例２で得たモノクローナル抗体を含む培養上清又は対照実験として非免疫マウス血清をTBS中に種々の濃度(非免疫マウス血清は100倍)に希釈した溶液中に室温下、１時間浸せきしたのち、TBSに１０分間浸せきし、洗浄する操作を２回繰り返した。続いて切片を、ウサギ抗マウス〔IgG＋IgM〕－ホースラディッシュパーオキシダーゼ複合体溶液(400倍希釈)中に３０分間浸せきしたのち、TBS洗浄を前記のように３回行った。次いで、0.05%ジアミノベンジジン(DAB)及び0.01%の過酸化水素を含有するトリス緩衝液(pH7.6)中に室温下、５分間浸せきしたのち、蒸留水で洗浄した。

次に、このようにして処理された切片を、光学顕微鏡での観察のためにヘマトキシリンで短時間染め、脱水したのちオイキットで封入した。

また、電子顕微鏡を用いた観察のために、前記のDABとの反応後、２%のグルタルアルデヒド溶液で切片を固定し、さらに１%四酸化オスミウムで後固定して脱水したのち、エポン(EPON)に包埋した。樹脂が固まってからウルトラミクロトーム(LKB社製)を用いて超薄切片を作成し、８０KVの条件で、日本電子社製JEM200CXを用いて観察した。

光学顕微鏡下での観察により、実施例１(４)で得た６０クローンの培養上清の老人斑特異性を評価した結果、最も強く反応するクローンS-1を選択したこのクローンS-1が産生するモノクローナル抗体をSA-1と名づける。

モノクローナル抗体SA-1は、定型老人斑(核がはっきりしている老人斑)及び原始老人斑(核がはっきりしない)のいずれにも強く反応し、ジアミノベンジジン顆粒によって深かっ色に強く染まった。しかし、脳切片中のミエリン(myelin)、軸索〔アクソン(axon)〕、神経細胞〔ニューロン(neuron)〕、グリア(glia)細胞とは全く反応しなかった。また、脳血管とは一部反応したが、これはSDATに随伴して起る脳血管へのアミロイド物質の沈着アミロイ

ドアンジオパチー(Amyloid Angiopathy)によるものと思われる。すなわちこのアミロイドアンジオパチーのアミロイドタンパクが老人斑アミロイドタンパクと極めて高い相同性を有するため、モノクローナル抗体SA-1が老人斑と共に一部の脳血管アミロイドと反応したものと思われる。

一方、電子顕微鏡下での観察によると、老人斑のアミロイド繊維は、暗色のパーオキシダーゼ-DAB反応生成物で覆われているのに対し、他の組織、すなわちグリア細胞やアルツハイマー原線維変化をもつ神経細胞及び軸索、アルツハイマー原線維変化を含まない神経細胞及び軸受、ミエリン、血管はすべて染まらなかった。

また、抗原タンパクを抽出した原発性アミロイドーシスの脾切片を、前記の脳切片の場合と同様にしてモノクローナル抗体SA-1と処理したが全く反応しなかった。

実施例3 モノクローナル抗体の精製

(1) 培養による方法

クローンS-1をウシ胎児血清10%含有DMEM培地を用いて、細胞濃度0.5×10^6~2×10^6個/mℓで培養し、24時間ごとにその培養上清を回収した。この回収した培養上清は、0.1Mリン酸緩衝液(pH8.0)に対して4℃で一晩透析してpHを8.0に調整した。

この液をウサギ抗マウスIgM抗体(マイルス社製、μ鎖特異的)を結合したセファロース-4B(ファルマシア社製)を充てんしたカラムに流し、培養上清中のモノクローナル抗体SA-1を該セファロース-4Bに結合させた。次いで、カラムに、0.1Mリン酸緩衝液(pH8.0)を流して十分に洗浄後、グリシン-塩酸緩衝液(0.1Mグリシン、0.2M塩化ナトリウムを含有する液に塩酸を加えてpH3.0に調整した液)を流し、溶出されるタンパク分画を回収した。回収したタンパク分画は、ただちに0.5Mリン酸緩衝液(pH7.2)を加えて中性にし、これを精製モノクローナル抗体(以下SA-1Pと略す)溶液とした。

(2) マウス腹腔による方法

あらかじめ腹腔にプリスタン(アルドリッチ社製)0.5mℓを注入して刺激しておいたマウス(6週令、BALB/C、雌)の腹腔に、5×10^6個の新規雑種細胞クローンS-1を注入した。およそ1週間後より腹水が貯留しだした。適宜注射器によって腹腔にたまった腹水を採取した。

このようにして得た腹水は、次のようにして精製を行った。腹水をまず3000rpmで20分間遠沈して沈殿を除去した。次に、得られた上清に10mℓ当り硫酸ナトリウム1.8gを加えて2時間室温で振とうし、1時間室温で静置して塩析した。塩析により生じた沈殿を8000×gで20分間遠沈して回収した。回収した沈殿を0.02Mリン酸緩衝液(pH6.8、0.05M塩化ナトリウムと0.02%アジ化ナトリウムを含む)で溶解し、一晩透析した。この液を次に、上記リン酸緩衝液にて平衡化したDEAE-セファデックスカラム(DEAE-Sephadex A-50、ファルマシア社製)に流して分画した。各分画の抗特異抗原(実施例1で用いた抗原)モノクローナル抗体活性を実施例1で述べた酵素免疫測定法によって測定し、抗特異抗原モノクローナル抗体活性を有する分画を回収した。

この分画はIgM溶出位置に一致した。これを精製モノクローナル抗体溶液とした。

実施例4 本発明のモノクローナル抗体(SA-1)の生化学的性質

(1) タンパク変性剤を加えた実施例1で、二次元電気泳動によって得た精製モノクローナル抗体SA-1Pを、まず、タンパク変性剤不存在下で等電点電気泳動し、次いでタンパク変性剤存在下に二次元電気泳動を行った。

すなわち、抗IgM抗体を結合したアフィニティカラムで精製したモノクローナル抗体SA-1P溶液(タンパク量1mg/mℓ)5μℓを、ポリアクリルアミド・チューブゲル(ゲルサイズ径3mm×6.5mm)に加え、0.01Mリン酸と0.14N水酸化ナトリウムとを用いて、pH3.5~10の間で等電点電気泳動した(定電圧200V、120分)。

次に、このチューブゲルを1重量%のSDSを含む4~17重量%のポリアクリルアミド濃度勾配をもつスラブゲル(ゲルサイズ横75×縦60×

厚さ2.7mm)上に密着させたのち、0.1%のSDSを含むトリス・グリシン緩衝液1(トリスヒドロキシメチルアミノメタン0.05M、グリシン0.384M、pH8.3)を用いて、定電流(ゲル当り30mA)で、約180分間泳動した。なお、泳動時間はブロムフェノールブルー(BPB)と結合したアルブミンの泳動状態より判断した。

このゲルを、クーマシー・ブリリアント・ブルー0.025重量%、メタノール50重量%、酢酸7重量%を含む水溶液(以下染色液と略す)に浸して、8時間室温でゆっくり振りまぜたのち、10%メタノール及び7%酢酸を含む脱色液を加えて1日間振りまぜて脱色した。

分子量マーカーを泳動した結果と、アルブミンの泳動位置とから、このモノクローナル抗体SA-1Pの分子量を決定した。

その結果、SA-1Pは、分子量170,000(160,000~180,000)と分子量900,000(800,000~1,000,000)の2つのバンドに分かれた。170,000のものは単量体であり、900,000のものは5量体であると考

えられる。また、等電点は単量体、5量体とも6.3~8.3であった。

(2) タンパク変性剤を加えない二次元電気泳動

(1)で用いた精製モノクローナル抗体SA-1P溶液について、電気泳動緩衝液及びポリアクリルアミドゲルにSDSを加えないこと以外は、

(1)と同様にして二次元電気泳動を行った。

その結果アルブミンの最先端部の移動度を1.0として、SA-1Pの移動度は0.16~0.57であった。

(3) モノクローナル抗体のクラスの決定

(1)と同じようにして、二次元電気泳動を行ったのち、以下のようにして転写を行った。

すなわち、転写用容器(イムノメディカ社製、商品名 水平型電気泳動式トランスファー・ブロッティング装置)にトリス・グリシン緩衝液2(トリスヒドロキシメチルアミノメタン0.025M、グリシン0.192M、pH8.3)を"おさえパット"が浸るまで入れたのち、おさえパットの上にろ紙をのせた。このろ紙の上に、二次元電気泳動したポリアクリルアミドスラブゲルをのせ、さらに、その上にニトロセルロース膜〔シュライハーアンドシュエル社製、75×55mmサイズに切って使用〕を重ねた。これらに一定電圧(20V)で18分間通電して、二次元電気泳動法で泳動・分画したモノクローナル抗体SA-1を、二次元電気泳動ゲルからニトロセルロース膜へ転写した。

このニトロセルロース膜(以下、転写ニトロセルロース膜という)を2%ウシ血清アルブミンを含むトリス・塩酸緩衝液(10mMトリスヒドロキシメチルアミノメタン・塩酸緩衝液、pH7.2、0.8%NaCℓ、0.01%NaN_3)中に浸し、一晩4℃で静置した。次に、転写ニトロセルロース膜を、ヤギ抗マウスIgM抗血清(μ鎖特異的、マイルス社製)をトリス・塩酸緩衝液で5倍希釈した液に浸して、室温(20~25℃)で1時間振りまぜ(20回/分)反応させた。反応後、転写ニトロセルロース膜をトリス・塩酸緩衝液に浸して室温で振とう(20回/分)することによって洗浄した。この際トリス・塩酸緩衝液は、5分おきに5回交換した。洗浄後、ペルオキシダーゼ標識ウサギ抗ヤギIgG

抗体(L+H鎖特異的、マイルズ社製を、1%ウシ血清アルブミンを含むトリス・塩酸緩衝液によって、あらかじめ決定しておいた示適濃度に希釈(通常500倍)した液(2次抗体液)に、転写ニトロセルロース膜を浸して室温で2時間静置し、反応させた。次に、上記と同様にして転写ニトロセルロース膜を洗浄したのち、酵素基質溶液を加えて室温で10分間静置し、転写ニトロセルロース膜上のペルオキシダーゼ活性を測定した。ここで酵素基質溶液には、0.2mM 3,3'-ジアミノベンジジン、30%過酸化水素水を0.1%含むトリス・塩酸緩衝液を用いた。

この結果、二次元電気泳動で得た2つのバンドが抗マウスIgM抗体と反応していることが分った。

同様にして、市販の抗マウスIgG、抗マウスK鎖、抗マウスλ鎖(いずれもマイルス社製、ウサギ血清、製造元能書に記された希釈倍率で使用)などの抗血清を用い、イムノブロッティングを行ったが、モノクローナル抗体SA-1Pは、抗マウスK鎖とのみ反応した。これらのことからモノクロー

ナル抗体SA-1PのクラスはIgM、タイプはK型であることが分った。

4．図面の簡単な説明

第1図及び第2図は、本発明のモノクローナル抗体SA-1を用いてSDAT患者の脳切片を検索した際の光学顕微鏡による観察図であり、第1図は定型老人斑、第2図の網目状老人斑の場合である。

第3図は老人斑と該モノクローナル抗体SA-1との反応の電子顕微鏡による観察図である。

第4図と第5図は精製モノクローナル抗体SA-1Pの二次元電気泳動結果であり、第4図はSDS存在下でのクーマーシー染色したもの、第5図はSDS存在下で泳動後、抗マウスIgM抗体を用いてイムノブロッティングしたものである。

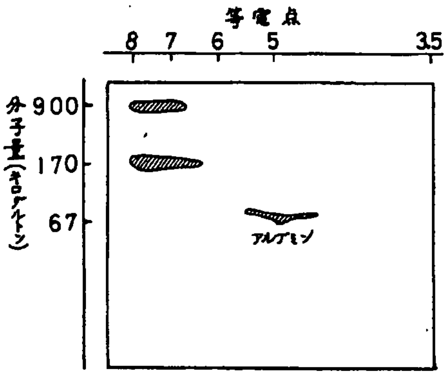
図面の浄書(内容に変更なし)

第1図

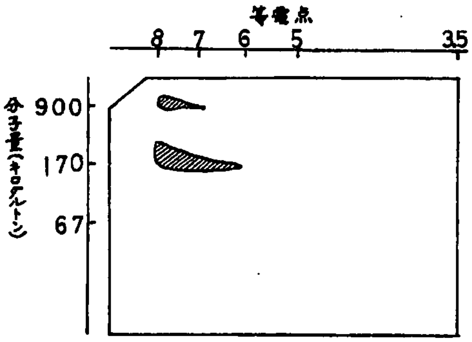
第2図

第3図

第 4 図


等電点
8
7
6
5
3.5
分子量(キロダルトン)
900
170
67
アルブミン

第 5 図


等電点
8
7
6
5
3.5
分子量(キロダルトン)
900
170
67

手続補正書(方式)

昭和61年8月15日

特許庁長官　黒田明雄　殿

1．事件の表示

昭和61年特許願第109433号

2．発明の名称

老人斑反応性モノクローナル抗体、それを産生する細胞株及び該モノクローナル抗体の製造方法

3．補正をする者

事件との関係　特許出願人

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電話(591)9910番

5．補正命令の日付　昭和61年7月2日
(発送日：昭和61年7月29日)

6．補正の対象　図面

7．補正の内容　添付図面中第1図及び第2図を別紙のとおり訂正します。

特許庁
61. 8. 15

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KAWARABAYASHI TAKESHI

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(54) TRANSGENIC ANIMAL FOR ALZHEIMER'S DISEASE MODEL

(57)Abstract:

PURPOSE: To provide the transgenic animal capable of being utilized for testing the effect of a medicine relating to an ability for reducing the characteristic parameters of the Alzheimer's disease, such as a β-protein antibody-resistant reactive substance formed in the brain of an animal.

CONSTITUTION: An Alzheimer's disease model transgenic animal comprises a human being-excluding mammalian into each of whose body cells or genital cells a recombined cell containing (1) the DNA sequence of a β-actin promoter, (2) the DNA sequence of a cytomegalovirus enhancer, (3) a DNA sequence coding the signal peptide of a human β-amiloid precursor protein, and (4) a DNA sequence coding the C-terminal peptide of the human β-amiloid precursor protein has been inserted, the C-terminal peptide having the length of 99-103 amino acid residues.

LEGAL STATUS

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審査請求　未請求　請求項の数6　ＦＤ　（全 17 頁）

(21)出願番号　特願平5－306026

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最終頁に続く

(54)【発明の名称】　アルツハイマー病モデルトランスジェニック動物

(57)【要約】

【構成】　1）ベータアクチンプロモーターのＤＮＡ配列、
2）サイトメガロウイルスエンハンサーのＤＮＡ配列、
3）ヒトベータアミロイド前駆体蛋白のシグナルペプチドをコードするＤＮＡ配列、および
4）ヒトベータアミロイド前駆体蛋白の９９ないし１０３個のアミノ酸残基長のＣ末端ペプチドをコードするＤＮＡ配列を含むことを特徴とする組み換え遺伝子が、体細胞および生殖細胞に組み込まれた、ヒト以外の哺乳動物であるアルツハイマー病モデルトランスジェニック動物。

【効果】　本発明のトランスジェニック動物は、動物脳内に形成される抗ベータ蛋白抗体反応性物質等のアルツハイマー病特有のパラメーターを減少させる能力に関する薬剤の効果検定に利用できる。

【特許請求の範囲】

【請求項１】　１）ベータアクチンプロモーターのＤＮＡ配列、
２）サイトメガロウイルスエンハンサーのＤＮＡ配列、
３）ヒトベータアミロイド前駆体蛋白のシグナルペプチドをコードするＤＮＡ配列、および
４）ヒトベータアミロイド前駆体蛋白の９９ないし１０３個のアミノ酸残基長のＣ末端ペプチドをコードするＤＮＡ配列を含むことを特徴とする組み換え遺伝子が、体細胞および生殖細胞に組み込まれた、ヒト以外の哺乳動物であるアルツハイマー病モデルトランスジェニック動物。

【請求項２】　ヒトベータアミロイド前駆体蛋白のシグナルペプチドが、配列表の配列番号１のペプチドである請求項１のアルツハイマー病モデルトランスジェニック動物。

【請求項３】　１）ベータアクチンプロモーターのＤＮＡ配列、
２）サイトメガロウイルスエンハンサーのＤＮＡ配列、および
３）ヒトベータアミロイド前駆体蛋白の９９ないし１０３個のアミノ酸残基長のＣ末端ペプチドをコードするＤＮＡ配列を含むことを特徴とする組み換え遺伝子が、体細胞および生殖細胞に組み込まれた、ヒト以外の哺乳動物であるアルツハイマー病モデルトランスジェニック動物。

【請求項４】　ヒトベータアミロイド前駆体蛋白のＣ末端ペプチドが、
１）配列番号２のアミノ酸配列を有する、正常ヒトベータアミロイド前駆体蛋白のペプチド、
２）配列番号３のアミノ酸配列を有する、２２番目のグルタミン酸がグルタミンに変換した変異体ペプチド、
３）配列番号４のアミノ酸配列を有する、４６番目のバリンがイソロイシンに変換した変異体ペプチド、
４）配列番号５のアミノ酸配列を有する正常ヒトベータアミロイド前駆体蛋白のペプチド、または
５）配列番号６のアミノ酸配列を有する、３番目のリジンがアスパラギンに、４番目のメチオニンがロイシンに変換した変異体ペプチドから選ばれる請求項１～３のアルツハイマー病モデルトランスジェニック動物。

【請求項５】　海馬領域において以下の組織病理学的特徴、
１）ベータアミロイド前駆体蛋白質Ｃ末端ペプチドの大量合成、
２）ＣＡ領域の海馬錐体細胞での神経細胞死、
３）グリア細胞の増加、および
４）異常リン酸化タウ蛋白質の沈着を有する、請求項１～４のアルツハイマー病モデルトランスジェニック動物。

【請求項６】　マウスである請求項１～５のアルツハイマー病モデルトランスジェニック動物。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】この発明は、一般的に疾患の治療に関連した薬の開発に有効な動物モデルに関するものである。更に詳しくは、ベータアミロイド前駆体蛋白（β－amyloid precursor protein, APP：以後、これをＡＰＰと呼ぶ）の一部をコードする外来性遺伝子構築体を自らのゲノムに取り込んだトランスジェニック（transgenic）動物の作製に関するもので、その中で、外来性遺伝子構築体をどの細胞タイプでも広く過剰発現させるよう仕組まれている。

【０００２】

【従来の技術】最近の発生工学の発展により、外来性遺伝物質（ＤＮＡ）を胚の核内へ注入するか感染させることにより、胚の染色体にその物質を組み込んだ胚（いわゆる形質転換胚）を作ることができるようになった(Gordon J. et al., Proc. Natl. Acad. Sci., USA, vol.77, p.7380-7384, 1980；Jaenisch R. et al., Cell, vol.32, p.209-216, 1983)。この胚は、仮親（里親）に移植することにより、成長させることができ、得られた成体動物は外来性DNAを自らの染色体に取り込んでおり、且つそれを発現することができる。形質転換された個体は、一般的にトランスジェニック動物と呼ばれる(Gordon J. and Ruddle F., Science, vol.214,p.1244-1246, 1981)。取り込まれた外来性DNAは、トランスジーン（transgene）と呼ばれ、一般的にプロモーターとｃＤＮＡ等の目的遺伝子とから成る。外来性ＤＮＡの発現は、成体にならずとも発現させることができ、例えば、胚の細胞分裂過程でも発現する場合がある。従って、その発現の結果、外来性DNAによりコードされる蛋白が生産され、特にその蛋白が生体にとって重要な機能を果たしているなら、その個体の発生のある時点では、個体の表現型に何らかの変化を引き起こすこともあり得る。表現型の変化を与える様式としては、個体で発現させるべき目的蛋白の過剰発現か、内在性の目的蛋白の発現の抑制があり、それを制御するのは、目的蛋白をコードする遺伝子の上流側に置かれているプロモーターかエンハンサーである。尚、発現抑制の仕方としては、アンチセンス法(Katsuki M. et al., Science, vol.241, p.593-595, 1988)等が挙げられる。

【０００３】個体が外来性DNAで形質転換された、あるいはその結果、本来の表現型が変わったという報告は、これまでに多くされており、特にPalmiter R.D. and Brinster R.L. (Annu. Rev. Genet., vol.20, p.465-499, 1986) や Gordon J.W. (Int. Rev. of Cytobiol., vol.115, p.171-229, 1989) 等の総説に詳しく述べられている。このトランスジェニック動物は、1) 発生過程での遺伝子発現のin vivoでの解析、2) 遺伝病の克服または軽減に向けた研究等の分野で利用されうる。外来性Ｄ

ＮＡによる胚の形質転換は、外来性ＤＮＡを外から与えることにより、最終的に胚核内の染色体内のDNA配列の一部に組み込まれることにより達成される。これを達成するにはいくつかの方法があるが、例えば、外来性DNAを微小ピペットに吸入し、これを１細胞期の前核内へ注入する方法、所謂、顕微注入法(Gordon et al., 1980)が一般的である。

【０００４】ＤＮＡを注入された胚はついで、偽妊娠雌の生殖道（輸卵管または子宮）に移植することにより、１個の生命体へと発生させることができる。この生命体は後日、外来性ＤＮＡを自らの染色体に取り込んでいるかどうか、ＰＣＲ法やサザンブロット法等で解析されることとなる。もしこの取り込みが確認されたら、この動物はin vivoにおける遺伝子発現解析（例えば、ノーザンブロット法や免疫抗体法等による解析）に利用される。この発現により、ヒトのある遺伝病に似た形質を引き起こすことも可能である。

【０００５】アルツハイマー病には、一説では後に述べるように、APPの過剰発現が関連すると考えられている（Terry R.D. and Katsman R, Ann. Neurol. vol.14, p.496-506, 1983）。アルツハイマー病患者脳には、アルツハイマー病特有の神経原線維変化［neurofibrillay tangles (NFT)、paired helical filaments (PHF) :以後これをＰＨＦと呼ぶ］、老人斑（neuritic plaque または senile plaque）及び脳アミロイド（amyloid）の沈着があり、脳アミロイドはＡＰＰから生じるからである。しかも、最近では、後で述べる家族性アルツハイマー病や遺伝性脳血管アミロイドアンギオパチー（amyloid angiopathy）でＡＰＰの遺伝子異常が発見されたことや、脳内に沈着したアミロイドの主成分であるアミロイドプラークコア蛋白（amyloid plaque core protein；APCP）あるいは、アミロイドコア蛋白（β－amyloid core protein）（後日、このポリペプチドは ベータ蛋白またはβ／Ａ４蛋白と命名される。以後、これをβ／Ａ４蛋白と呼ぶ）自体が神経毒性を有するという報告（Yankner B.A. et al., Science, vol.245, p.417-420, 1989）等から、ＡＰＰからβ／Ａ４蛋白が代謝され、沈着する機序の解明がアルツハイマー病の病因解明の最も本質的なアプローチと考えらている。しかし、残念ながら、これまでアルツハイマー病のモデルとなる動物が知られていないか、あるいは、確立されていないため、このような仮説を立証する手立てがなかった。そこで、人為的にＡＰＰ遺伝子を組み込ませたトランスジェニック動物を作り、このＡＰＰをトランスジェニック動物脳内で過剰発現させ、アミロイド沈着を引き起こした結果、最終的にアルツハイマー病に似た動物、所謂ヒトアルツハイマー病モデルができる可能性がある。

【０００６】最近、ヒトのＡＰＰｃＤＮＡの全長あるいは一部分を過剰発現させることにより、脳内のアミロイド沈着を引き起こしたトランスジェニックマウスがいく

つかの研究室から相次いで報告された（Kawabata S. et al., Nature, vol.345, p.476-478, 1991；Quon D. et al., Nature, vol.352, p.239-241, 1991；Wirak D.O. et al., Science, vol.253, p.323-325, 1991）。しかし、Kawabata らの報告は、その後、追試に成功せず、論文は撤回された（Nature, vol.356, p.265,1992）。更に、Ｗｉｒａｋらの報告も、その形質変化はトランスジーンによるものではないということである（Science, 28, Feb., 1992）。特許出願でもいくつかのアルツハイマー病モデルトランスジェニック動物を確立したという報告が、例えばＷＯ９３／１４２００、ＷＯ９３／０２１８９、ＷＯ９２／１３０６９、ＷＯ９２／０６１８７、ＷＯ９１／１９８１０、ＥＰ４５１７００及びＷＯ８９／０６６８９として公開されているが、いずれも遺伝子の構築だけであったり、得られたトランスジェニック動物で単にＡＰＰの沈着がみられたといった間接的な証拠しかない。従って、現在のところ、はっきりとしたアルツハイマー病モデル動物は確立されていないと思われる。これに対し、本発明で作出されたトランスジェニック動物は、アルツハイマー病に伴う様々な症状と類似した形質を示しており、この意味では本発明で作出されたトランスジェニック動物は、アルツハイマー病の発症原因の解明のための実験系を提供し、また、アルツハイマー病発症の阻止、あるいは、アルツハイマー病発症に伴う神経細胞死等を阻止するようなアルツハイマー病治療薬をスクリーニングするための系を提供し得るものと言える。

【０００７】前にも述べたように、アルツハイマー病と関連した形態学的、病理学的変化としては、ＰＨＦの形成及び脳アミロイドの沈着の２点がある。ＰＨＦはアルツハイマー病以外の神経性疾患にも出現するが、神経と神経の間に見られるアミロイド沈着、所謂、老人斑及び脳血管周囲にも沈着するアミロイド沈着は、アルツハイマー病特異的と考えられている。特に、老人斑は高齢者のダウン症候群患者脳（アルツハイマー病も発症させている）でも見られる。老人斑アミロイドを構成する主要蛋白は、部分的に精製され、約４．２ｋＤの３９～４２個のアミノ酸から成るβ／Ａ４蛋白から成ることが判明した（Glenner G. and Wong C.W., BBRC, vol.120, p.1131-1135, 1984）。このアミノ酸配列は決定され（Glenner G. and Wong C.W., 1984；Masters C.R. et al., Proc. Natl. Acad. Sci., USA, vol.82, p.4245-4249, 1985）、そのアミノ酸配列はこれまで報告されている蛋白のものと全く異なるものであった。

【０００８】近年、ヒト胎児脳組織ｃＤＮＡライブラリーよりβ／Ａ４蛋白を含む比較的大きなサイズの蛋白（前駆体）をコードするｃＤＮＡが単離され、そのＤＮＡ配列解析から、６９５個のアミノ酸より成る（この蛋白をＡ６９５という）こと、β／Ａ４蛋白は、アミノ酸配列５９７－６９５の位置に相当することがわかった

（Kang J. et al., Nature, vol.325, p.733-736, 1987）。更に、Ａ６９５以外にも、もっと大きいサイズのＡＰＰｃＤＮＡ（A751、A770）が報告された（Kitaguchi et al., Nature, vol.331, p.530-532, 1988)。このＡ７５１蛋白はＡ６９５に５６個分のアミノ酸が付与されたもので、この特殊なインサートは、クニツファミリー（Kunitz family）のセリンプロテアーゼインヒビター（serine protease inhibitor）（以下ＫＰＩと呼ぶ）に非常に高い相同性を示す（Kitaguchi et al., 1988）。一方、Ａ７７０蛋白は、Ａ７５１の５７個のインサートのすぐ後にＭＲＣ ＯＸ－２抗原に相同性の高い１９個分のアミノ酸が挿入されたタイプである。これらＡ７５１、Ａ７７０は、全身臓器に多く発現している。尚、この３者は、同一の遺伝子（ＡＰＰ）からアルターナティブスプライシング（alternative splicing）によって生じることが示されており（Kitaguchi et al., 1988; Ponte P. et al., Nature, vol.331, p.525-527, 1988; Tanz R. et al., Nature, vol.331, p.528-530, 1988）、いずれもＣ末端から９９番目の間にβ／Ａ４蛋白部分を有すること（２８アミノ酸は細胞膜外に、１１～１４アミノ酸は細胞膜内に存在）から、脳内のアミロイド沈着に関係する蛋白をコードするものと考えられる。

【０００９】アルツハイマー病患者脳におけるＡＰＰの脳内局在を、ＡＰＰのいくつかの部位に対する数種の抗体を作製し、免疫組織化学的に検討すると、老人斑等が染められることが解かった（Wong C.W. et al., Proc. Natl. Acad. Sci., USA, vol.82, p.8729-8732, 1985; Allsop D. et al., Neurosci. Letter, vol.68, p.252-256, 1986; Shoji M. et al., Brain Res., vol.512, p.164-168, 1990a; Shoji M. et al., Am. J. Pathol., vol.137, p.1027-1032, 1990b; Shoji M. etal., Brain Res., vol.530, p.113-116, 1990c）。従って、アルツハイマー病患者脳で見つかった老人斑を構成するアミロイド蛋白はこれら抗体により認識されると言える。これらの抗体を用いて、例えば、ＡＰＰをある動物の脳内で強制発現させた場合、ＡＰＰとその代謝分画蛋白の神経における局在性を追跡することができる。

【００１０】ＡＰＰは全身臓器に広範に発現する蛋白であり、また、蛋白進化の過程でもよく保存された蛋白である（マウスとヒトでは、アミノ酸レベルで97%の一致が見られる）ことから、細胞間接着（cell-cell adhesion）や細胞分化等に重要な役割を果たしているものと想定されたが（Shivers B.D. et al., EMBO. J., vol.7, p.1365-1370, 1988）、正確な役割は未だに不明である。最近、β／Ａ４蛋白が未分化な海馬神経細胞に対し、低濃度では神経栄養因子として働く一方、成熟した神経細胞に対しては、高濃度では神経毒性として働くことが示され、注目されている（Yankner B.A. et al., 1989）。この実験系では、栄養因子と神経毒性として働く部分は、β／Ａ４蛋白の２５～３５番目のアミノ酸に相当し、この部分はタヒキニン系のペプチドと相同性があることが示された（Yankner B.A etal., 1989）。これに関連して興味深いのは、β／Ａ４蛋白を大脳皮質または海馬内へ注入すると、この現象がin vivo においても起こること、及びＰＨＦの構成成分である異常燐酸化タウ（tau）蛋白の生成が誘導された点である（Kowall N.W. et al., Proc. Natl. Acad. Sci., USA, vol.88, p.7247-7251, 1991）。従って、β／Ａ４蛋白蓄積とＰＨＦ生成の深い関連が示唆された。更に、もっと最近では、ＡＰＰのC末端側の細胞内部位は、プロテイキナーゼＣやＣａ$^{2+}$／カルモジュリン依存性プロテイキナーゼIIにより燐酸化され（Gandy S. et al., Proc. Natl. Acad. Sci., USA, vol.85, p.6218-6221, 1988）、また、細胞膜直下に存在する主要ＧＴＰ結合蛋白であるGOとAPPが相互作用するという報告（Nishimoto I et al., Nature, vol.362, p.75-78, 1993）もあり、ＡＰＰがシグナル伝達にも関与する可能性が指摘されている。

【００１１】ＡＰＰ遺伝子はヒトの場合、第２１染色体の長腕に存在することが知られている（Goldgaber D. et al., Science, vol.235, p.877-880, 1987）。近年、家族性アルツハイマー病（familial Alzheimer's disease）のうち、早期発症型（発症年齢が６５歳以下のもの）家系において、ＡＰＰのアミノ酸番号６４２（Kang J. et al., 1987の配列に基づく；以下、ＡＰＰの塩基配列、アミノ酸配列はKang J. et al., 1987に基づいて表記してある）にＶａｌからＩｌｅへの突然変異が発見された（Goate A. et al. , Nature, vol.349, p.704-706, 1991; NaruseS. et al., Lancet, vol.337, p.978-979, 1991; Yoshioka K. et al., BBRC, vol.178, p.1141- 1146, 1991; Hardy J. et al., Lancet, vol.337, p.1342-1343, 1991）。更に、同じアミノ酸部位にＰｈｅ、Ｇｌｙという他の アミノ酸への突然変異が発見され（Murrell J. et al., Science, vol.254, p.97-99, 1991; Chartier-Harlin M-C et al., Nature, vol.353, p.844-846, 1991）、家族性アルツハイマー病の発症にこのValの変異が重要な役割を果たしていると考えられている。また、オランダ型（Dutch-type）の遺伝性脳出血に伴うアミロイド沈着の場合、β／Ａ４蛋白の内部、即ちＡＰＰのアミノ酸番号６１８にＧｌｕからＧｌｎへの突然変異がみられる（Levy E. et al., Science, vol.248, p.1124-1126, 1990）。更には、β／Ａ４蛋白のＮ末端側の２個のアミノ酸の変異（アミノ酸番号５９５のＬｙｓがＡｓｎへ、及びアミノ酸番号５９６のＭｅｔがＬｅｕへ変換）がスウェーデンの家族性アルツハイマー病と関連することが提唱され（Mullan M. et al., Nature Genet, vol.1, p.345-347, 1992）、このタイプはスウェーデン型突然変異と呼ばれる。このようにAPPに関する分子生物学的解析は進んだが、アルツハイマー病患者脳で何故アミロイドが蓄積され、沈着するか、そして、β／Ａ４蛋白の蓄積によりどのように神経細胞が変

性していくのかというメカニズムについては何ら有効な情報は未だない。

【００１２】現在、最も問題となっているのは、脳内にアミロイド沈着が生じるためには、ＡＰＰのどのような代謝経路に問題があるのかで、詳細に検討され始めている。例えば、ＡＰＰｃＤＮＡを導入したヒト胎児性腎臓（embryonic kidney）細胞２９３株から、膜結合型C末端分画９ｋＤを抽出し、そのN末端のアミノ酸配列を決定した結果、β／Ａ４蛋白部分のN末端から１６番目のＬｙｓでＡＰＰが切断されていることが解かった（Esch F.S. et al., Science, vol.248, p.1122-1124,1990）。しかし、脳アミロイドとして沈着するには、ＡＰＰはβ／Ａ４蛋白部分のN末端とC末端で切断され凝集することが必要であり、Ｅｓｃｈらの明らかにした代謝系では不溶性のβ／Ａ４蛋白は生成されて来ない。この点から更に、様々な代謝系の関与やその異常等が推定されているが、未だ明らかな結果は得られていない。現在のところ、ＡＰＰのプロセッシングには、1）ＡＰＰをβ／Ａ４蛋白部分の１５番目のアミノ酸で終わる分子量１００ｋＤ以上の分泌性派生体（secretedderivative）とＣ末端側の低分子産物とに分解される、いわゆる分泌経路（secretary pathway）と、2）β／Ａ４蛋白の部分を全長の形で含むC末端側の様々な大きさのペプチドを生成する、いわゆるエンドゾーマル／リソゾーマル経路（endosomal/lysosomal pathway）の２つがあると考えられている（Golde T.E. et al, Science, vol.235, p.728-730, 1992）。

【００１３】従って、上記家族性アルツハイマー病型、オランダ型、及びスウェーデン型のＡＰＰ遺伝子上の３箇所の突然変異が生じた際に、これらの２つの代謝経路はどのように影響されるのかという点はまだ解決されていないが、これらＡＰＰアナログはＡＰＰのプロセッシングの経路をエンドゾーマル／リソゾーマル経路に積極的に変える構造を持つものと想定される。この意味では、これらＡＰＰアナログを過剰発現するトランスジェニック動物システムは、ＡＰＰのプロセッシング機構を明らかにするための有用な材料を提供するものである。

【００１４】

【発明が解決しようとする課題】本発明は、ＡＰＰの合成に関する分子機構、詳しくは、ＡＰＰ合成後のＡＰＰのプロセッシングに関する分子機構解明する方法を提供するものである。更に重要なことは、β／Ａ４蛋白の合成と沈着を阻止するための薬剤のin vivoスクリーニング系を提供するものである。

【００１５】

【課題を解決するための手段】アルツハイマー病に関連したＡＰＰをコードするＤＮＡ断片を哺乳動物好ましくはマウスの１細胞期胚の前核に顕微注入し、次いでこの注入された胚は、偽妊娠メスに移植され、トランスジェニック動物出産へと至る。このトランスジェニック動物は、アルツハイマー病に関連するＡＰＰ蛋白を過剰発現すると考えられる。注入されたＤＮＡには、トランスジェニック動物の非特異的で様々なタイプの細胞で目的の蛋白を発現せしめるようなプロモーターが含まれている。β／Ａ４蛋白はＡＰＰのＣ末端側領域から生成されるので、ＡＰＰのＣ末端側領域のみの大量発現は、β／Ａ４蛋白の形成を促進し、その結果として、特有の神経の退化及び老人斑の形成等が始まると考えられる。

【００１６】本発明の重要な点は、β／Ａ４蛋白を含むＡＰＰのＣ末端側領域を強力なプロモーターの制御の下、神経やそれ以外のどのタイプの細胞でも過剰発現させることができる点である。そして、その結果、アルツハイマー病特有の海馬におけるアミロイドの沈着、燐酸化タウ蛋白の出現、グリア細胞の増加、海馬付近における神経細胞死、更には、動物の活発的な行動の低下が引き起こされる点である。また、内在性のＡＰＰｍＲＮＡのアルターナティブスプライシングのパターンの変化もこの発現によって引き起こされる点である。更に、本発明の重要な点は、β／Ａ４蛋白の中で最底1箇所アミノ酸の置換を持つアナログをコードするＤＮＡ配列を有するトランスジェニック動物を作出した点である。これにより、ＡＰＰのエンドゾーマル／リソゾーマル経路におけるプロセッシングに変化をきたし、プロテアーゼの活性特異性が変わるような蛋白が生じる可能性があり、その結果、脳内での大量のアミロイドの蓄積が望まれる。従って、本発明で紹介されたトランスジェニック動物は、ＡＰＰ蛋白とプロテアーゼとの相互作用、あるいは、内在性ＡＰＰと導入された外来性蛋白同志の相互作用等をin vivoで研究するための有用な系を提供するものであり、また、アルツハイマー病治療薬の探索にも利用できるものである。

【００１７】本発明の目的は、β／Ａ４蛋白に相当するＡＰＰのＣ末端側領域、及びそのアナログを神経細胞や他の細胞に強力に発現せしめるために必要なＤＮＡ配列、所謂、組み換えＤＮＡを有するトランスジェニック動物を提供することであり、本発明の有用性としては、このトランスジェニック動物がアルツハイマー病の病因解析及びアルツハイマー病治療薬のin vivo スクリーニングのために用いることができることである。本発明の特徴としては、このトランスジェニック動物がＡＰＰの大量合成、燐酸化タウ蛋白の出現、グリア細胞の増加、神経細胞死等の一連のアルツハイマー病特有の症状を示すため、これまで知られているＡＰＰ遺伝子導入トランスジェニックマウスに比べ、よりアルツハイマー病に近い動物モデルであると言える。本発明をより具体的に説明するため、以下に実施例を示す。

【００１８】

【実施例】次に掲載する実施例は、ＤＮＡ配列、融合（fusion）構築体、トランスジェニックマウス等をどのように作るか等を完全に開示し、記載することを目的と

したものだが、これによって本発明が実施例に限定されるものではない。

【0019】実施例1

トランスジェニックマウスで発現されるべきプラスミドｐβＡ／ＮＯＲβ、ｐβＡ／ＦＡＤβ、ｐβＡ／Ｄβ、ｐβＡ／ΔＮＯＲβ及びｐβＡ／ＮＬβの構築マウスで発現させるべき目的遺伝子は以下のように作成した。正常なヒトＡＰＰｃＤＮＡのシグナルペプチド（アミノ酸番号１番目から１７番目）とβ／Ａ４蛋白に相当するＡＰＰのＣ末端側（アミノ酸番号５９７番目から６９５番目）との融合遺伝子（これをＮＯＲβと呼ぶ）をHorton R.M. et al (Gene, vol.77, p.61-68, 1989) の方法に習い合成した。先ず、ヒト脳ｐｏｌｙ (A) ＲＮＡ（#6516-1；Clontech社）を材料とし、ＲＴ－ＰＣＲ法によりヒト脳ｃＤＮＡライブラリーを合成した。用いたプライマー (primer) は、リバースプライマーＢＡＰＰ－６（配列番号７）、センスプライマーＢＡＰＰ－７（配列番号８）、センスプライマーＢＡＰＰ－１０（配列番号９）、リバースプライマーＢＡＰＰ－１２（配列番号１０）で、これらを適当に組み合わせて用いることにより、ＮＯＲβを合成した。合成されたＮＯＲβは、２％アガロースゲルによる電気泳動で分画し、単離した。このＮＯＲβは、ＸｂａＩ消化後、ｐＧＥＭ３Ｚ(－) (Promega社) のＸｂａＩ部位へ導入し、この組み替えプラスミド (pGEM3Z／NORb) を大腸菌内にて増幅させ、ジデオキシ鎖終止法 (dideoxy chain-termination) (Sanger et al., Proc. Natl. Acad. Sci., USA, vol.74, p.5463-5468, 1977) によるシークエンシングを行い、ＮＯＲβ配列が目的通り、正しいことを確認した。

【0020】Ｄβ（配列番号１と配列番号３の結合したもの）及びＦＡＤβ（配列番号１と配列番号４の結合したもの）は、基本的にＮＯＲβと同じ構造であるが、ＤβはＡＰＰアミノ酸番号６１８にＧｌｕからＧｌｎへの変異を持つオランダ型の遺伝性脳出血アミロイドアンギオパチーで、ＦＡＤβはＡＰＰアミノ酸番号６４２においてＶａｌからＩｌｅへの変異を持つ家族性アルツハイマー病に各々対応する。また、Ｄβ及びＦＡＤβは、そのＡＰＰｃＤＮＡ (Kang J. et al., 1987) ３′側の非コード領域約３０ｂｐを含んでいる。これらは、前記ヒト脳ｃＤＮＡライブラリーを基にＨｏｒｔｏｎらの方法に従い、ＰＣＲ法で目的の配列を増幅した。Ｄβの場合、ＢＡＰＰ－１０、ＢＡＰＰ－６、ＢＡＰＰ－７の他、リバースプライマーＢＡＰＰ－８（配列番号１１）、センスプライマーＢＡＰＰ－２（配列番号１２）、およびリバースプライマーＢＡＰＰ－１５（配列番号１３）を用いた。ＦＡＤβの場合、ＢＡＰＰ－１０、ＢＡＰＰ－６、ＢＡＰＰ－７の他、リバースプライマーＢＡＰＰ－３（配列番号１４）、センスプライマーＢＡＰＰ－９（配列番号１５）およびＢＡＰＰ－１５を用いた。

【0021】ΔＮＯＲβ（配列番号５のペプチドのＮ端にＭｅｔが付加したもの）は、１７個のアミノ酸から成るシグナルペプチドがない点を除けば、基本的にＮＯＲβと同じ構造である。ΔＮＯＲβはセンスプライマーＢＡＰＰ－１３（配列番号１６）およびＢＡＰＰ－１２を用い、ｐＧＥＭ３Ｚ／ＮＯＲβに対しＰＣＲを行い、インサートを増幅させた。増幅された断片はｐＧＥＭ３Ｚにクローニングされ、次いで、シークエンシングを行い、その配列の正しさを確認した。ＮＬβ（配列番号６のペプチドのＮ端にＭｅｔが付加したもの）はΔＮＯＲβと同様、センスプライマーＢＡＰＰ－１４（配列番号１７）およびＢＡＰＰ－１２を用い、ｐＧＥＭ３Ｚ／ＮＯＲβに対しＰＣＲを行い、インサートを増幅させた。増幅された断片はｐＧＥＭ３Ｚにクローニングされ、シークエンシングを行い、その配列の正しさを確認した。ＮＬβはスウェーデン型の遺伝性アルツハイマー病に対応するもので、ＡＰＰのアミノ酸番号５９５のＬｙｓがＡｓｎへ、及びアミノ酸番号５９６のＭｅｔがＬｅｕへ変異している。

【0022】一方、上記標的遺伝子を発現させるためのベクターを以下のように作成した。ニワトリベータアクチンプロモーターとその上流にサイトメガロウイルスエンハンサーを有する哺乳動物発現ベクターｐＣＡＧＧＳ (Niwa H. et al., Gene, vol.108, p.193-200, 1991) より２．３ｋｂ断片をＳａｌＩ／ＰｓｔＩ消化により切り出し、これをクローニングベクター pBluescript (Stratagene社)の ＳａｌＩ／ＰｓｔＩ部位へ挿入し、ｐＢｓＣＡＧ－２ベクターを構築した（図１）。この２．３ｋｂ断片の中には、上記エンハンサー／プロモターの他に、ウサギベータグロビン遺伝子の一部（第２イントロン、第３エクソン、３′側非コード領域から成る）が含まれている。通常、ｃＤＮＡ等の発現させたい目的遺伝子は、第３エクソンのＥｃｏＲＩ部位に挿入される。このｐＢｓＣＡＧ－２のＥｃｏＲＩ部位に上記目的遺伝子ＮＯＲβ、ＦＡＤβ、Ｄβ、ΔＮＯＲβ、及びＮＬβの各種ＤＮＡ断片を挿入し、トランスジェニックマウス発現用プラスミドｐβＡ／ＮＯＲβ、ｐβＡ／ＦＡＤβ、ｐβＡ／Ｄβ、ｐβＡ／ΔＮＯＲβ、及びｐβＡ／ＮＬβを構築した（図２）。マウス１細胞期胚へのＤＮＡ導入には、これら融合構築体よりＳａｌＩ／ＢａｍＨＩ消化によりトランスジーンを単離し、これを用いた。

【0023】尚、この構築には、ＤＮＡを消化したり、つなげたり、ＤＮＡ断片を単離する等の操作が行なわれたが、このためには Maniatis T. et al (Molecular Cloning, A. Laboratory Manual, 1982) の標準的ＤＮＡ組み換え技術が用いられた。また、インサートの結合部周辺のＤＮＡ配列は、シークエンシングにより確認された。

【0024】実施例２　１細胞期胚の回収及びそれへのＤＮＡ導入

１細胞期胚は、既に雄と交配してあったＢ６Ｃ３Ｆ１雌マウスの輸卵管より回収した。回収した胚は前核期の初期ステージにあり、この時点では雄核と雌核とがまだ分離されており、容易に識別されうる。回収された胚からは周囲に存在する卵胞細胞（cumulus cell)等を除去し、適当に洗浄後、ＤＮＡ注入まで一時、３７℃、５％ＣＯ$_2$空気条件下で保存される。好ましくは、３０mm径バクテリオロジカルディッシュ（bacteriological dish）(No.333656, Nunc社）上、Ｍ１６培養液（Whittingham D.G., J. Reprod. Fert., vol.4, p.7-21, 1971) ５０μｌ ｄｒｏｐ（パラフィン油で覆われる）で保存される。トランスジーンを含む融合構築体は、上述の方法で調製した。上述のいかなる融合構築体も、クローニングされ得、ここで述べる方法に習い、１細胞期胚に導入することができる。

【００２５】次に、主にＮＯＲβ発現ベクター（ｐβＡ／ＮＯＲβ）から得られるＤＮＡの導入について詳細に解説するが、ｐβＡ／ＮＯＲβ以外の他の融合構築体にも通用できる方法である。まず最初に、ｐβＡ／ＮＯＲβは宿主大腸菌にクローニングされ、大量増幅の後、抽出された。更に精製するため、塩化セシウムによる超遠心、続く 臭化エチジュウム（ethidium bromide）の除去後、透析処理に付された。こうして精製されたプラスミドは、適当な制限酵素による消化（この場合、ＳａｌＩとＢａｍＨＩが用いられた）、続く０.８％アガロースゲル電気泳動により、目的のトランスジーンを単離す＊＊ることができる。こうして得られたトランスジーンは、外径が約1mmの注入ピペットを使い、１細胞期胚前核へ顕微注入される（Hogan B. etal., Manipulating the Mouse Embryo, 1986)。トランスジーンを含む約１０μｌのＤＮＡ溶液（約２０００コピーのトランスジーンが含まれる）を吸引し、雄性前核へこのDNAを注入する。注入を受けた胚は、数時間か1日間培養に付され、次いで偽妊娠Ｄａｙ１（プラグ確認日をＤａｙ１と判定する）のＩＣＲ仮親マウス輸卵管内へ移植される。移植を受けた仮親マウスは胎仔を出産するまで飼育される。出産後、新生児は離乳までの１月間、仮親マウスより保育を受け、次いで、尾DNAのサザンブロット解析に付される。トランスジェニック動物と判定されたＦ０マウス（founder）は、他の非トランスジェニックマウスと交配させ、そのＦ１子孫を得た後（それらは卵や精子の形で凍結保存される）、生後１０〜３０週目にてすべて殺処分し、ノーザンブロット解析、病理学的解析に付した。

【００２６】表１には、一つの例として、βＡ－ＮＯＲβトランスジーンを マウス１細胞期胚に注入して得られた結果が示されている。この中にはＤＮＡ注入後、移植されたマウス胚の数、注入された胚の移植後の生存胎仔の数、実際トランスジェニック動物と判定されたＦ０マウス胎仔の数、等が表示されている。

【００２７】

【表１】

βＡ－ＮＯＲβトランスジーンを有するトランスジェニックマウスの生産効率

トランスジーン	生まれた新生仔の数／移植した胚の数（%）	トランスジェニックマウスの数（%）
βＡ－ＮＯＲβ	１２０/５６０（２１）	３５（２９）

【００２８】表１に示されるように、生まれた１２０匹のマウスうち、３５匹は、トランスジェニック動物であった。これらのマウスを飼育した結果、１匹（０３０４）は、生後約１０週目でその活発な動きが低下し、もう１匹（１１０２）は水頭症を発していた。他のトランスジェニック動物は、生後１０〜３０週目までは、正常様であった。これらは、すべて採材されるまでには、その配偶子が取られ、凍結保存に付された。そして、ノーザンブロット、ウエスタンブロットによりスクリーニングを行ない、発現の強い５系統（０２０２、０３０４、１００２、１１０２、１３０１）を選択した、。以下の解析は、主にこの５系統に関するものである。

【００２９】実施例３　トランスジーン由来のmＲＮＡ発現

トランスジーン由来のmＲＮＡ発現は、０３０４、１１０２等を含むβＡ－ＮＯＲβトランスジェニックマウスの系統においてノーザンブロット解析を行なった。トランスジェニック動物及び非トランスジェニックマウスの脳を含む各種臓器より、全ＲＮＡを単離し、２０μｇの全ＲＮＡを１.１％アガロース／１.１Ｍホルムアルデヒドゲル電気泳動に付し、次いで、ナイロン膜フイルターに移した。プレハイブリダイゼーションは、ハイブリダイゼーション液［５ＸＳＳＣ（１ＸＳＳＣ＝０.１５Ｍ ＮａＣｌ、１５mM Na-citrate, ｐＨ７.４）、５０％ホルムアミド、５mM ＥＤＴＡ、５mｇ／mｌの変性サケＤＮＡ、 ５X Denhardt試薬等を含む］の中で、４２℃にて２時間行った。次いで、ランダムにプライミングしたｃＤＮＡプローブ（ＮＯＲβ断片）を熱変性させた後、これをハイブリダイゼーション液に加え、ハイブリダイゼーションを行った。反応は４２℃にて１８時間行った。洗浄は、０.１ＸＳＳＣ／０.１％ＳＤＳ中、５６℃、２０分間行った。フィルターは－８０℃にて、２４〜７２時間暴した（増感スクリーン＋コダックＸＡＲ－５フイルム使用）。ノーザンブロット解析の一例を図３に示す。図３には、選択されたトランスジェニック動物系統のうち、比較的発現の強い０３０４、１１０２、

及び、非トランスジェニック動物のサンプルが示されている。調べた臓器は、脳、肝、腎臓、小腸、精巣である。脳では、０３０４では、内在性のＡ６９５mRNA（～３.４ｋｂ）の量に比べ、～１０倍程高いトランスジーン由来mRNAの発現（～１ｋｂ）があるが、１１０２はそれと比べると、やや低い。非トランスジェニック動物は、トランスジーン由来mＲＮＡの発現は全く見られない。同じ傾向は、他の臓器にも見られる。また、興味深いことに、トランスジェニックマウスでは、Ａ６９５mＲＮＡが増加している（例えば、１１０２及び０３０４の肝サンプルを参照）とか、Ａ６９５mＲＮＡの他にＡ７５１（～３.８ｋｂ）、及びＡ７７０（～３.８５ｋｂ）mＲＮＡも出現しているのが判かる（例えば、１１０２及び０３０４の脳サンプルを参照）。おそらく、外来性のＮＯＲβの過剰発現の結果、内在性のＡＰＰmＲＮＡのアルターナティブスプライシングのパターンが何らかの理由で変化を起こした結果と思われる。

【００３０】実施例４　ウェスタンブロット解析

βＡ－ＮＯＲβトランスジェニック動物及び非トランスジェニック動物（コントロール）のマウス脳内のＡＰＰ蛋白発現様式をウェスタンブロット解析により決定した。蛋白ホモジネイトを、Shivers B.D.ら（1988）の方法に基づき、脳全てあるいは３／４から調製した。５０μｇ量のサンプルを１０／１６％Tris-Tricine SDSゲルにて電気泳動に付し、イモビルム－Ｐメンブレン（Immobilm-P membrane）へエレクトロブロッティング（electroblotting ）により転写させた。ブロットを抗ＡＰＰ抗体Ｗ６１Ｃ［ＡＰＰのＣ末端側ペプチド（６６０番目から６９５番目）に対するウサギ抗体；Shoji M. et al., 1990c］（1／500希釈）と反応させ、ＥＣＬ（Amersham社）システムによる非RI法による検出システムを用い、ＡＰＰ蛋白の検出を試みた。

【００３１】ウェスタンブロット解析の一例を図４に示す。図４には、１１系統のトランスジェニックマウスと非トランスジェニック動物の結果が示されている。即ち、これまで報告されている哺乳動物ＡＰＰ isoformの平均的サイズに相当する約１２０－ｋｂ近傍のいくつかのバンドがコントロール及びトランスジェニックマウスの脳及び各種臓器の蛋白ホモジネイト中に検出される。更に、今回発現させたトランスジーン由来の蛋白（１１.４ｋＤ）がコントロールサンプルと比べ顕著に増加している。特に、高い発現（５～６倍）は、０２０２、１００２、１３０１トランスジェニック動物サンプルに見られる。しかしながら、この方法では、β／Ａ４蛋白に相当する～４.２ｋＤのバンドは検出されず、この蛋白は調べたトランスジェニック動物脳では作られていないか、あっても少ないだろうと考えられる。

【００３２】実施例５　抗体を用いたマウス脳の免疫組織化学的解析

トランスジーンの発現を組織、細胞レベルで詳細に解析するため、トランスジェニック動物及び非トランスジェニック動物から得た脳を含む各種臓器に対し、抗ＡＰＰ抗体を用いた免疫組織化学的染色を行なった。調べたマウスは、βＡ－ＮＯＲβを有する3系統のトランスジェニックマウス、及び非トランスジェニックマウスである。マウスをペントバルビタールで麻酔後、脳を含む各種臓器を摘出し、４％パラホルムアルデヒド（ＰＢＳが溶媒）にて１日間固定後、パラフィンへ包埋し、５μm厚の切片を作製した。切片は脱パラフィン後、脱水させ、０.５％過ヨーソ酸にて３０分処理後、正常山羊血清でブロッキングを行い、適当に希釈された抗体（１／500）と反応させた。反応は、室温、３時間行ない、次いでビオチン化された抗ウサギＩｇＧと反応させ（室温、２時間）、更にアビジン－ビオチンパーオキシダーゼ複合体（avidin-biotin peroxidase complex : ABC）と反応させた。これらの反応は、製造者（ＡＢＣキット；ベクター社, Burlingame, USA)の推薦する方法で行なった。パーオキシダーゼは、3,3′-diamino benzidine（DAB)／$NiCl_2$にて発色、可視化される。切片の核染色は、メチルグリーンにて行なった。時に、切片を抗体で反応させる前に、切片はニッスル（Nissul）染色に付された。

【００３３】βＡ－ＮＯＲβ－０３０４系統のトランスジェニック動物脳切片と非トランスジェニック動物脳切片とをニッスル染色で観察すると、このトランスジェニック動物系統においては、神経細胞死が見られた。特に海馬付近のＣＡ３領域を中心とした海馬錐体細胞での神経の変性、脱落が著しい。図５にその写真を示す。βＡ－ＮＯＲβ－０３０４系統のトランスジェニック動物脳切片と非トランスジェニック動物脳切片とを抗ＡＰＰ抗体の一つＷ６１Ｃで染色すると非トランスジェニック動物脳切片に比べ、トランスジェニック動物脳では、免疫反応は、大脳皮質、海馬等の神経細胞に特に強く見られた。更に陽性反応は、多数の神経突起に見られた。しかし、中脳、脳幹、小脳等には、ほとんど見られなかった。同じような所見は、他の抗体［Ｗ６３Ｎ；ＡＰＰのＮ末端側のペプチド（１８番目から３８番目）に反応する抗体：Shoji M. et al., 1990c］を用いても見られた。図６にその写真を示す。

【００３４】βＡ－ＮＯＲβ－０３０４系統のトランスジェニック動物脳切片と非トランスジェニック動物脳切片とをグリア細胞（astrocyte）を特異的に染色する抗ＧＦＡＰ（glial fibrillary acidic protein）抗体と反応させると、トランスジェニック動物脳では非トランスジェニック動物に比べ、大脳皮質、海馬、前脳基底部に著明なグリア細胞の増加を認めた。図７にその写真を示す。グリア細胞の増加は、アルツハイマー病に相関するとされ（Beach T. G. et al., Glia, vol.2,p.420-436, 1989)、おそらく図５から推測されうるに、神経細胞の死滅後、グリア細胞がその後を埋める形で増殖したと

考えられる。βA－NORβ－0304系統のトランスジェニック動物脳切片と非トランスジェニック動物脳切片とを異常燐酸化タウ蛋白と特異的に反応する抗体β1－28（Ihara Y. et al., Nature, vol.304, p.727-730, 1983）で切片を反応させると、トランスジェニック動物脳では、この抗体と反応する構造物が海馬付近に認められた［図8(A)］。このような陽性反応は非トランスジェニック動物脳では認められない［図8(B)］。

【0035】トランスジェニックマウスの全身写真を図9に示す。図9の(A)はβA－NORβ－0304トランスジェニックマウス（写真中央）及び非トランスジェニックマウス（写真上方）の全身写真であり、(B)はβA－NORβ－0304トランスジェニックマウスの全身写真である。

【0036】

【発明の効果】本発明のトランスジェニックマウスは、マウス脳内に形成される抗ベータ蛋白抗体反応性物質、抗異常燐酸化タウ蛋白抗体反応性物質等のアルツハイマー病特有のパラメーターを減少させる能力に関する薬剤の効果検定に利用できる。例えば、検定されるべき薬剤は対照動物、即ち本発明のトランスジェニック動物でない動物群及び本発明のトランスジェニック動物に同時に＊投与される。この薬剤は、動物の脳内に上記パラメーターに影響を及ぼすのに充分な期間、あるいは神経細胞死を抑えるのに充分な期間を越えて連続的に投与され得るだろう。この充分な期間を経て薬剤を投与された後、トランスジェニック動物及び対照の非トランスジェニック動物は、供試され、脳内の解析が行われる。そして、上記パラメーターを比較することにより、薬剤の効能についてひとつの決定を下すことができる。

【0037】

【配列表】

配列番号：1
配列の長さ：51
配列の型：核酸
鎖の数：二本鎖
トポロジー：直鎖状
配列の種類：cDNA to mRNA
起源
生物名：ヒト（homo sapiense）
配列の特徴：
他の情報：ヒトベータアミロイド前駆体のシグナルペプチド

配列

```
ATG CTG CCC GGT TTG GCA CTG CTC CTG CTG GCC GCC TGG ACG GCT CGG     48
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1               5                  10                  15
GCG                                                                 51
Ala
```

【0038】配列番号：2
配列の長さ：297
配列の型：核酸
鎖の数：二本鎖
トポロジー：直鎖状
配列の種類：cDNA to mRNA
起源
生物名：ヒト（homo sapiense）
配列の特徴：
他の情報：ヒトベータアミロイド前駆体のC末端ペプチド

配列

```
GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA     48
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
  1               5                  10                  15
TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT     96
Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
             20                  25                  30
GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC    144
Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr
         35                  40                  45
TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT GTG    192
Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val
     50                  55                  60
GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC CAC CTG TCC AAG    240
Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys
 65                  70                  75                  80
```

```
ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG TTC TTT GAG CAG     288
Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln
                85                  90                  95
ATG CAG AAC                                                         297
Met Gln Asn
        99
```

【0039】配列番号：3

配列の長さ：297

配列の型：核酸

鎖の数：二本鎖

トポロジー：直鎖状

配列の種類：cDNA to mRNA

起源

生物名：ヒト (homo sapiense)

配列の特徴：

他の情報：ヒトベータアミロイド前駆体のC末端ペプチド

配列

```
GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA      48
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
  1               5                  10                  15
TTG GTG TTC TTT GCA CAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT      96
Leu Val Phe Phe Ala Gln Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
             20                  25                  30
GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC     144
Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr
         35                  40                  45
TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT GTG     192
Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val
     50                  55                  60
GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC CAC CTG TCC AAG     240
Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys
 65                  70                  75                  80
ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG TTC TTT GAG CAG     288
Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln
                85                  90                  95
ATG CAG AAC                                                         297
Met Gln Asn
        99
```

【0040】配列番号：4

配列の長さ：297

配列の型：核酸

鎖の数：二本鎖

トポロジー：直鎖状

配列の種類：cDNA to mRNA

起源

生物名：ヒト (homo sapiense)

配列の特徴：

他の情報：ヒトベータアミロイド前駆体のC末端ペプチド

配列

```
GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA      48
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
  1               5                  10                  15
TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT      96
Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
             20                  25                  30
GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG ATC ATC ATC ACC     144
Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Ile Ile Thr
         35                  40                  45
```

```
TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT GTG     192
Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val
     50                  55                  60
GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC CAC CTG TCC AAG     240
Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys
 65                  70                  75                  80
ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG TTC TTT GAG CAG     288
Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln
                 85                  90                  95
ATG CAG AAC                                                         297
Met Gln Asn
         99
```

【0041】配列番号：5
配列の長さ：３０９
配列の型：核酸
鎖の数：二本鎖
トポロジー：直鎖状
配列の種類：cDNA to mRNA
起源
生物名：ヒト（homo sapiense)
配列の特徴：
他の情報：ヒトベータアミロイド前駆体のC末端ペプチド

配列

```
GAA GTG AAG ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT      48
Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
  1               5                  10                  15
CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA      96
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
             20                  25                  30
GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG     144
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
         35                  40                  45
ATC GTC ATC ACC TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT     192
Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
     50                  55                  60
CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC     240
His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
 65                  70                  75                  80
CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG     288
His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
                 85                  90                  95
TTC TTT GAG CAG ATG CAG AAC                                         309
Phe Phe Glu Gln Met Gln Asn
            100         103
```

【0042】配列番号：6
配列の長さ：３０９
配列の型：核酸
鎖の数：二本鎖
トポロジー：直鎖状
配列の種類：cDNA to mRNA
起源
生物名：ヒト（homo sapiense)
配列の特徴：
他の情報：ヒトベータアミロイド前駆体のC末端ペプチド

配列

```
GAA GTG AAT CTG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT      48
Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
  1               5                  10                  15
```

CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA 96
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
20 25 30
GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG 144
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
35 40 45
ATC GTC ATC ACC TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT 192
Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
50 55 60
CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC 240
His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
65 70 75 80
CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG 288
His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
85 90 95
TTC TTT GAG CAG ATG CAG AAC 309
Phe Phe Glu Gln Met Gln Asn
100 103

【0043】配列番号：7
配列の長さ：26
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　＊
＊起源：なし
生物名：なし
株名：なし
配列の特徴：リバースプライマーDNA。BAPP—6と名付けた。

配列
TTCTGCATCC GCCCGAGCCG TCCAGG 26

【0044】配列番号：8
配列の長さ：29
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　※
※起源：なし
生物名：なし
株名：なし
配列の特徴：センスプライマーDNA。BAPP—7と名付けた。

配列
GCTCGGGCGG ATGCAGAATT CCGACATGA 29

【0045】配列番号：9
配列の長さ：25
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　★
★起源：なし
生物名：なし
株名：なし
配列の特徴：センスプライマーDNA。BAPP—10と名付けた。

配列
CTCTAGAGAT GCTGCCCGGT TTGGC 25

【0046】配列番号：10
配列の長さ：30
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸
起源：なし
生物名：なし
株名：なし
配列の特徴：リバースプライマーDNA。BAPP—12と名付けた。

配列
GGCTCTAGAG CATGTTCTGC ATCTGCTCAA 30

【0047】配列番号：11
配列の長さ：21
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　＊
＊起源：なし
生物名：なし
株名：なし
配列の特徴：リバースプライマーDNA。BAPP－8と名付けた。

配列
GTCTTGTGCA AAGAACACCA A　21

【0048】配列番号：12
配列の長さ：21
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　※
※起源：なし
生物名：なし
株名：なし
配列の特徴：センスプライマーDNA。BAPP－2と名付けた。

配列
TTGGTGTTCT TTGCACAAGA T　21

【0049】配列番号：13
配列の長さ：24
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　★
★起源：なし
生物名：なし
株名：なし
配列の特徴：リバースプライマーDNA。BAPP－15と名付けた。

配列
GGATCCAACT TCAGAGGCTG CTGT　24

【0050】配列番号：14
配列の長さ：21
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　☆
☆起源：なし
生物名：なし
株名：なし
配列の特徴：リバースプライマーDNA。BAPP－3と名付けた。

配列
GGTGATGATG ATCACTGTCG C　21

【0051】配列番号：15
配列の長さ：21
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　◆
◆起源：なし
生物名：なし
株名：なし
配列の特徴：センスプライマーDNA。BAPP－9と名付けた。

配列
GCGACAGTGA TCATCATCAC C　21

【0052】配列番号：16
配列の長さ：38
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：他の核酸　＊
＊起源：なし
生物名：なし
株名：なし
配列の特徴：センスプライマーDNA。BAPP－13と名付けた。

配列
GGCTCTAGAG ATGGAAGTGA AGATGGATGC AGAATTCC　38

【0053】配列番号：17
配列の長さ：38
配列の型：核酸
鎖の数：一本鎖

トポロジー：直鎖状
配列の種類：他の核酸
起源：なし
生物名：なし ＊

＊株名：なし
配列の特徴：センスプライマーDNA。BAPP―14と名付けた。

配列
GGCTCTAGAG ATGGAAGTGA ATCTGGATGC AGAATTCC 38

【図面の簡単な説明】

【図1】サイトメガロウイルスエンハンサー／ニワトリベータアクチンプロモーターを有するプラスミドｐＢｓＣＡＧ−２のプラスミドマップを表した図である。

【図2】サイトメガロウイルスエンハンサー／ニワトリベータアクチンプロモーターとＮＯＲβ、Ｄβ、ＦＡＤβ、ΔＮＯＲβ及びＮＬβとを結合した各種トランスジーンを表わした図である。

【図3】βＡ−ＮＯＲβトランスジェニックマウス（１１０２，０３０４）及び非トランスジェニックマウスから採取した各種組織の全ＲＮＡのノーザンブロット解析結果を示した図である。分子量のサイズはＫで示され、分子量マーカーは図の右側に記されている。

【図4】βＡ−ＮＯＲβトランスジェニックマウス及び非トランスジェニックマウスから採取した脳抽出物のウェスタンブロット解析を示した図である。用いた抗体は抗ＡＰＰ抗体Ｗ６１Ｃである。

【図5】(A)はβＡ−ＮＯＲβ−０３０４トランスジェニック動物脳、そして(B)は非トランスジェニックマウス脳の海馬領域におけるニッスル染色を表わした写真である。

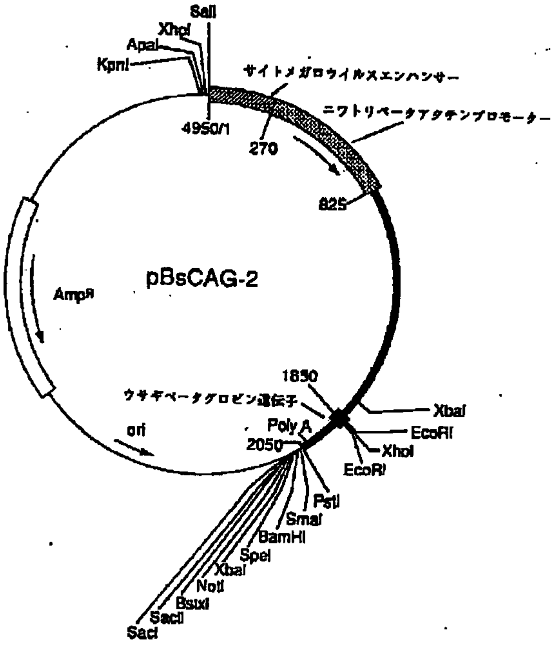
【図6】(A)はβＡ−ＮＯＲβ−０３０４トランスジェニック動物脳、そして(B)は非トランスジェニックマウス脳における抗ＡＰＰ抗体Ｗ６１Ｃによる免疫反応産物の顕微鏡写真である。

【図7】(A)はβＡ−ＮＯＲβ−０３０４トランスジェニック動物脳、そして(B)は非トランスジェニックマウス脳における抗ＧＦＡＰ抗体による免疫反応産物の顕微鏡写真である。

【図8】(A)はβＡ−ＮＯＲβ−０３０４トランスジェニック動物脳、(B)は非トランスジェニックマウス脳における抗タウ抗体β１−２８による免疫反応産物の顕微鏡写真である。

【図9】(A)はβＡ−ＮＯＲβ−０３０４トランスジェニックマウス及び非トランスジェニックマウスの全身写真であり、(B)はβＡ−ＮＯＲβ−０３０４トランスジェニックマウスの全身写真である。

【図1】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KpnI
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270
825
pBsCAG-2
AmpR
1850
ウサギベータグロビン遺伝子
Poly A
2050
ori
XbaI
EcoRI
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EcoRI
PstI
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NotI
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SacII
SacI

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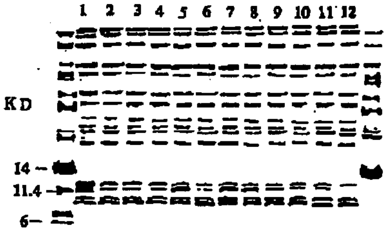
1 2 3 4 5 6 7 8 9 10 11 12
KD
14
11.4
6

1	トランスジェニック動物（０２０２）
2	トランスジェニック動物（０３０５）
3	トランスジェニック動物（０４０１）
4	トランスジェニック動物（０８０４）
5	トランスジェニック動物（１００２）
6	トランスジェニック動物（１００４）
7	トランスジェニック動物（１３０１）
8	トランスジェニック動物（１３０３）
9	トランスジェニック動物（１４０２）
１０	トランスジェニック動物（１５０１）
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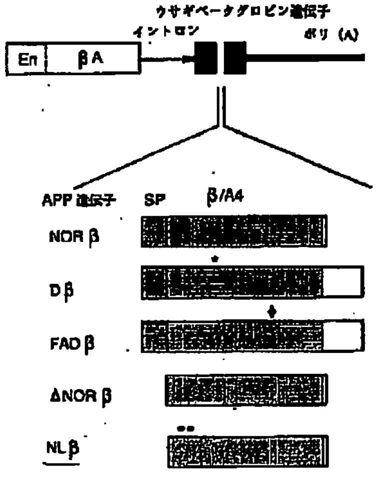
１２	非トランスジェニック動物

【図2】


ウサギベータグロビン遺伝子
イントロン
ポリ（A）
En
βA
APP遺伝子
SP
β/A4
NORβ
Dβ
FADβ
ΔNORβ
NLβ

En：サイトメガロウイルスエンハンサー

βA：ニワトリベータアクチンプロモーター

▨：APPコーディング領域

SP：シグナルペプチド

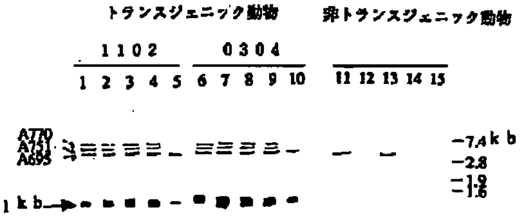
β/A4：β/A4蛋白に対応するAPPのC－末端領域

＊：コドン22番におけるGlu→Gln置換

♦：コドン50番におけるVal→Ile置換

●●：コドン3番におけるCys→Asn置換およびコドン4番におけるMet→Leu置換

【図3】


トランスジェニック動物
非トランスジェニック動物
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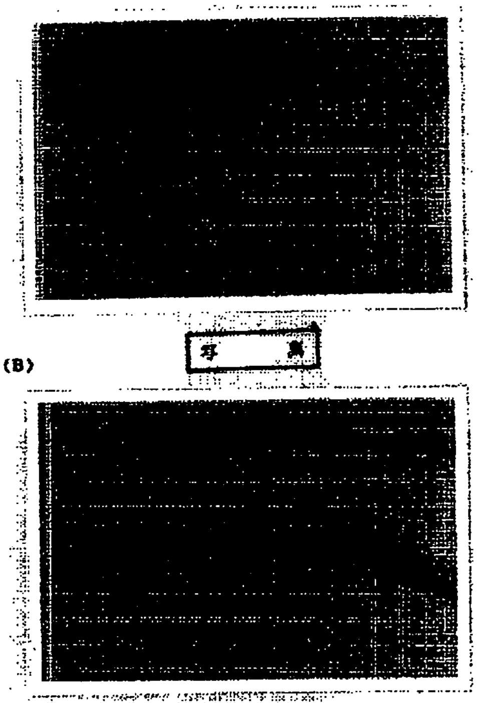
11 12 13 14 15
A770
A751
A695
1kb
7.4kb
2.8
1.9
1.6

1、　6、11	脳
2、　7、12	肝臓
3、　8、13	腎臓
4、　9、14	小腸
5、10、15	精巣

【図5】

図面代用写真

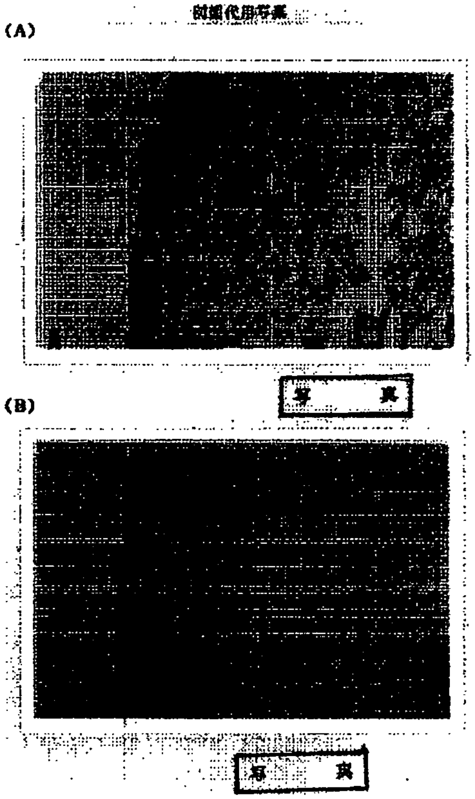


写　真

写　真

【図6】

図面代用写真



【図7】

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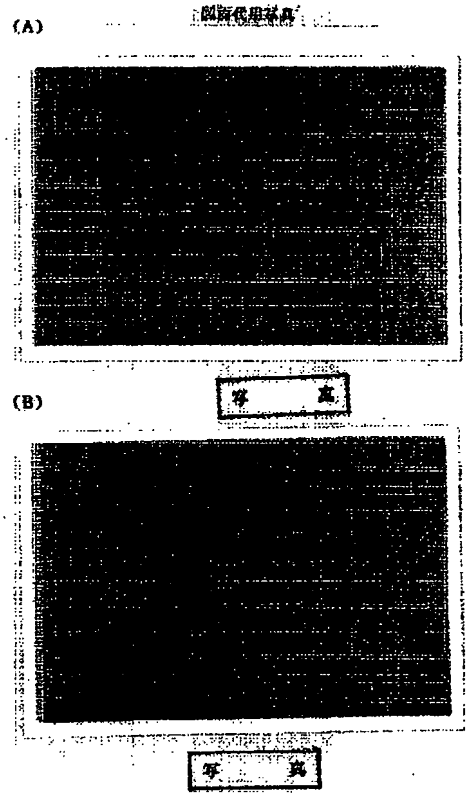

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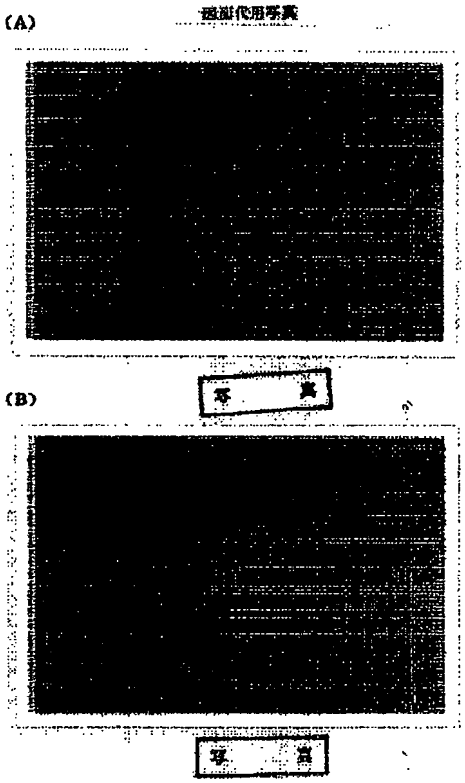
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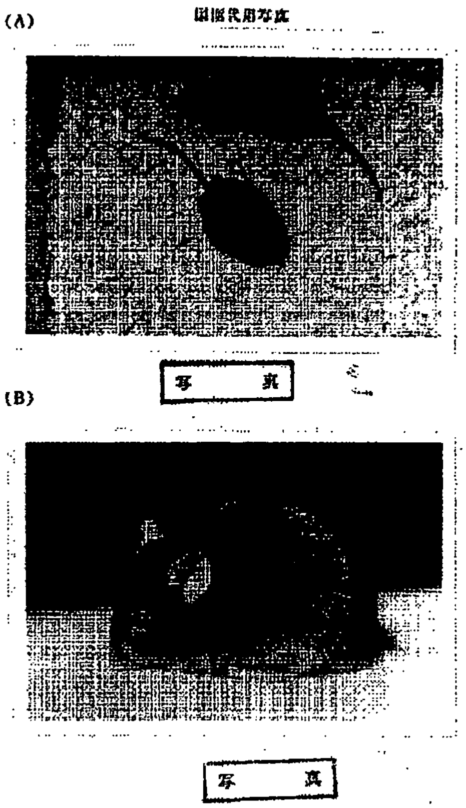
図面代用写真
写　真
(B)
写　真

【図9】


(A)
図面代用写真
写　真
(B)
写　真

フロントページの続き

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(57) Abstract

A gene, NACP, is disclosed along with its nucleotide and amino acid sequence. Also disclosed are diagnostic and therapeutic methods of utilizing the NACP nucleotide and polypeptide sequences. The NACP polypeptide is a precursor of NAC, a peptide associated with amyloid deposits in the brains of patients with typical neuropathological features of Alzheimer's disease (AD). Also disclosed is the amino acid sequence of NAC and of two contiguous fragments thereof, X and Y peptides. Diagnostic and therapeutic methods relating to amyloid disorders associated with NAC are also disclosed.

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NOVEL COMPONENT OF AMYLOID IN ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

STATEMENT OF GOVERNMENT SUPPORT

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RELATED U.S. PATENT APPLICATIONS

This application is a continuation in part of U.S. patent application serial no. 08/114,393, filed on August 30, 1993.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to diagnosis and treatment of neuronal abnormalities, in particular the deposition of amyloid plaques characteristic of Alzheimer's Disease.

Description of Related Art

The most common cause of disabling dementia in humans is Alzheimer's disease ("AD"). Its incidence increases sharply with age, and it is a major public health problem in our aging population. Persons suffering from Alzheimer's disease show a characteristic neuropathology, including synaptic loss, senile plaques and neurofibrillary tangles. Neurofibrillary tangles comprise paired helical filaments ("PHF") (D.L. Selkoe, *et al.*, *Science*, 235:873-876, 1987). A senile plaque commonly comprises a mass of disorganized neurites surrounding a deposit of extracellular filaments of an amyloid polypeptide called A4 or β amyloid protein ("Aβ").

Deposition of fibrillar deposits of Aβ a 39/43 residue amyloid, is considered the pathological hallmark of AD. Recently, molecular cloning based on the sequence of the Aβ protein indicated that it is encoded as part of a larger precursor (PreA4) that maps to chromosome 21 (Kang, *et al.*, *Nature*, 325:733-736, 1987; Goldgaher, *et al.*, *Science*, 235:877-880, 1987; Tanzi, *et al.*, *Science*, 235:880-884, 1987; Robakis, *et al.*, *Proc. Natl. Acad. Sci.* 84:4190-4194, 1987). There are three major alternatively spliced products of the amyloid mRNA (Ponte, *et al.*, *Nature*, 331:525-527, 1988,; Tanzi, *et al.*, *Nature*, 331:528-530, 1988; Kitaguchi, *et al.*, *Nature*, 331:530-532, 1988). The smallest of these products, the 695-residue precursor protein (PreA4$_{695}$), has been synthesized *in vitro* and shown to be a N-glycan membrane protein that spans the lipid bilayer once (Dyrks, *et al.*, *EMBO J.*, 7:949-957, 1988). Two other forms of PreA4 (PreA4 751 and PreA4 770) contain a 56 residue insert which has a protease-inhibitory function. The amyloidogenic A4 protein is derived in part from the transmembrane domain and from part of the adjacent extracellular domain. A precursor-product relationship has been demonstrated.

The A4 gene is expressed in brain and peripheral tissues, such as muscle and epithelial cells (Goeder, *EMBO J.*, 6:3627-3632, 1987; Bahmanyar, *et al.*, *Science*, 237:77-88, 1987; Zimmerman, *et al.*, *EMBO J.*, 7:1365-1370, 1988; Shivers, *et al.*, *EMBO J.*, 7:1365-1370, 1988), yet for reasons still unknown, the amyloid deposits in AD are confined to the brain.

Recently, *in situ* hybridization analyses were published that indicate an alteration of the amount of PreA4 mRNA in brains of AD patients when compared to normal individuals (Higgins, *et al.*, *Proc. Nat'l Acad. Sci. USA*, 85:1297-1301, 1988; Cohen, *et al.*, *Science*, 237:77-88, 1987; Lewis, *et al.*, *Proc. Nat'l Acad. Sci. USA*, 85:1691-169, 1988). These results implicate a role for gene regulation in AD.

In addition to Aβ, heparan sulfate proteoglycan, ferritin, immunoglobulins, and many acute phase proteins such as α-1 antichymotrypsin, apolipoprotein E, complements, serum amyloid P, and trace peptides have been reported to be associated with amyloid. However, supportive biochemical data demonstrating the presence of these proteins in amyloid preparations from the brains of Alzheimer's victims are not yet available, raising the possibility that these may not be intrinsic components of amyloid.

All forms of amyloid in amyloid deposits, including the Aβ, show a significant β-pleated sheet component (Snow, A.D., *et al.*, 1987). Yet the precursor of amyloid Aβ protein is soluble and does not exhibit a significant β-pleated sheet component. Recent studies of C. Haass, *et al., Nature*, 359:322-325, (1992); P. Seubert, *et al., Nature*, 359:325-327 (1992); M. Shoji, *et al., Science*, 258:126-129 (1992), have demonstrated that Aβ is generated and secreted from various types of cells under physiological conditions, implying that Aβ is soluble in aqueous solutions.

The physiological process responsible for changing the structure of the precursor protein is the subject of much enquiry. Recently a study by Roses, *et al.*, demonstrates that apolipoprotein E binds Aβ (*Proc. Natl. Acad. Sci. USA*, 90:1977-1981, 1993). Thus, apolipoprotein E may act as a molecular chaperone that mediates the β-pleated amyloid formation of Aβ as suggested by T. Wisniewski, *et al.* (*Neurosci. Lett.*, 135:235-238, 1992).

New and further information concerning the molecular biology involved in formation of amyloid deposits such as those found in Alzheimer's disease awaits discovery of additional intrinsic constituents associated with amyloid in the brains of those affected with Alzheimer's disease. On a physiological basis, recent studies have shown that amyloid deposition might be the result of aberrant processing of APP, and its abundance is an important parameter to consider in diagnosing the disease on a neuropathological basis. In addition, the cognitive dysfunction that characterizes AD is apparently attributable to synaptic loss (Terry, *et al.*, *Ann.Neurol.*, 30:572-580 (1993); Mattson, *et al.*, *TINS*, 16:409-414 (1993)). Recent studies strongly suggetsthat there is a connection between the abnormal processing of synaptic proteins and amyloid formation (Masliah, *et al.*, *Brain Path.*, 3:77-85 (1993)).

However, despite the knowledge that AD is related to neuritic plaques and synaptic loss, diagnosis of the disease is difficult. Currently, the only way of confirming the presence of these lesions in a living patient is by brain biopsy. However, this technique is rarely utilized because of the substantial risks to the patient involved in performing it. As a result, AD is usually diagnosed on the basis of clinical symptoms and the results of neuropsychological tests. Nonetheless, because AD can be mimicked by other disorders (such as depression), confirmation of an AD diagnosis often cannot be conclusively made until autopsy.

Several methods for *in vivo* diagnosis of AD have been proposed but have not yet yielded definitive results. One such approach attempts to detect amyloid and/or the precursor protein for it (APP) in blood and cerebrospinal fluid. These measurements have not, however, been shown to positively correlate to the development of neuritic plaques in AD. Another approach involves detection of a mutated form of the gene for the amyloid precursor protein. While the presence of this genetic alteration appears to be more predictive of AD than does circulating levels of amyloid and APP, the mutated gene is only found in some familial cases of AD. As a result, presence of the mutated gene would correlate to the onset of AD in less than 1% of all potential AD cases.

In vivo diagnosis of AD is further limited by the blood/brain barrier. Because of the barrier, detection of amyloid deposits by binding assays (and evaluation of synaptic loss associated with dementia) has been limited to autopsy studies (see, e.g., Masliah, *et al., Am. J. Pathol.*, 137:1293-1297, 1990 [quantitation of synapse loss in brain tissue section through use of labelled anti-synaptophysin antibodies]).

Further, the blood/brain barrier has also prevented (to date) the effective use of antibodies for *in vivo* diagnosis and therapy of AD. Thus, a promising *in vitro* use of a monoclonal antibody 10H3 which targets amyloid deposits (Majocha, *et al. J. Nucl. Med.*, 33:2184-2189) has not yet been extended to an *in vivo* application. Due to the size of antibodies like 10H3, there is some doubt whether they can successfully and innocuously cross the blood/brain barrier.

Clearly, a need exists for a noninvasive method for *in vivo* detection of amyloid deposits in brain tissue of patients who are suspected of having AD. In combination with present techniques for clinical diagnosis of AD, such a technique would be useful in confirming a diagnosis of, and evaluating the prognosis for, the disease.

SUMMARY OF THE INVENTION

Novel peptides obtained by purification and analysis of the amino acid sequences in an amyloid preparation of brain tissue obtained from patients with symptoms of Alzheimer's disease have been shown to be fragments of a novel amyloid component ("NAC"). Antibodies were raised against synthetically produced fragments of these novel peptides and used in immunohistochemical and electron microscopic analyses demonstrating that the peptides are localized in amyloid fibrils in AD brain tissue and are amyloidogenic.

Complementary DNA ("cDNA") encoding a 140 amino acid protein identified as the precursor ("NACP") of NAC is provided. NACP is a highly abundant synaptic protein, which degrades to form NAC. NAC is self-aggregating; i.e., it has a significant ability to bind to itself and become part of amyloid fibrils and neuritic plaque. The invention therefore provides NAC and NACP peptides useful as ligands to identify and quantify syanpses and plaques toward diagnosis and monitoring of diseases associated with synaptic loss and neuritic plaque formation, such as AD. The invention also provides methods for treatment of such diseases.

In particular, in the preferred diagnostic embodiment of the invention, a detectably labelled NAC/NACP peptide which will specifically bind NAC deposits in brain tissue, is administered parenterally to a mammal (preferably a human). Binding of the administered peptide to NAC/NACP in brain tissue is detected using suitable *in vivo* diagnostic imaging techniques. Most preferably, this detection will be by positron emission tomography (PET) or single photon emission computed tomography (SPECT).

In another aspect of the invention, the binding data generated as described above is evaluated with any clinical signs of a neuropsychological disorder to assist in confirmation or refutation of an initial diagnosis of AD. To the extent that the binding data reveals the extent of amyloid deposition, the data may also be used to evaluate the prognosis for a patient with a confirmed diagnosis of AD. Thus, the diagnostic method of the invention will provide physicians with valuable information concerning the medical status of a patient who is suspected of suffering from AD.

In another aspect of the invention, the binding data described above is evaluated with the results of tests for synapse loss in brain tissue to assist in confirmation of an AD diagnosis and to evaluate the prognosis for the patient. Data evidencing a relationship between amyloid deposition and synapse loss in AD brain tissue will also be of use in research toward understanding the etiology of AD.

In another aspect of the invention, labelled NAC/NACP peptides are utilized in *in vitro* studies of amyloid deposition in sections of brain tissue for use in confirming an AD diagnosis and/or for research purposes. For example, *in vitro* (and *in vivo*) use of detectably labelled NAC/NACP peptides may be used to evaluate agents to inhibit NAC formation, binding and deposition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 A shows immunohistochemical staining with anti-NAC antibodies of amyloid in diffuse, primitive, and mature plaques on slides of brain sections from patients with symptoms of Alzheimer's disease. In Panel A hippocampal sections were stained with anti-NAC antibody, anti-X1. In Panel B, in addition to amyloid staining, occasional staining of dystrophic neurites (arrows) was detected with a anti-NAC antibody, anti-Y. Absorption with the corresponding peptide eliminated the staining. Panel C shows absence of immunohistochemical staining by anti-sera to NAC on slides of AD brain sections when pre-absorbed with NAC peptides. Panel D shows an electron micrograph of specific staining by anti-X1 antibody on amyloid fibrils (arrows) in AD brain sections. Amyloid fibrils were also stained with anti-Y antibody.

Figure 2 A shows the nucleotide sequence of cDNA encoding the precursor of the NAC protein and the 140 amino acid sequence of the NAC precursor protein encoded by a 420 bp open reading frame with the X and Y fragments located contiguously in the middle of the precursor. The nearest in-frame stop codon (TAA) upstream to the putative initiation methionine codon is marked by an asterisk. The termination codon is marked by two asterisks. Sequence for X and Y peptides are boxed. Synthetic oligonucleotide mixtures used for PCR are indicated as lines above the corresponding cDNA. Polyadenylation signals are underlined.

Figure 2 B is a graph showing the hydropathy profile of the NAC precursor protein with the NAC region being the most hydrophobic.

Figure 3 is a Western blot of brain homogenate with anti-NAC polyclonal antibody anti-X1. NAC precursor protein in lane 4 is detected as a M_r 19K protein. The bacterially expressed NAC precursor protein expressed in *E. coli* transfected with pSENACP migrated in lanes 2 and 5. Lanes 1 and 6 contain *E. coli* transfected with pSE380 vector control; and lanes 3 and 4 show normal human brain. Lanes marked with (+) indicate X1 antibody was preabsorbed with X1 peptide; while those marked (-) indicated X1 antibody was preabsorbed with a control peptide. The arrow indicates NACP detected as a M_r 19K protein whose staining was blocked by preabsorption of the antibody with X1 peptide, thus showing specificity of the antibody.

Figure 4 A is a sequence listing of seven repeated sequence motifs in the NAC precursor amino acid sequence.

Figure 4 B is a sequence listing showing homology in the NAC precursor amino acid sequence at amino acids 48-56 and 70-78. Bold letters indicate the common amino acids among the repeat.

Figure 4 C is a comparison of the cDNA listings of EST01420 (EMBL/GenBank Libraries) and the NAC precursor showing homology therebetween at the N-terminal region of NAC.

Figure 5 is a Northern Blot of mRNA for NACP. Lane 1 shows normal adult midfrontal cortex (female, aged 88); lane 2 shows cerebellum from the same individual as lane 1; lane 3 shows fetal whole brain (female, 24 week fetus); lane 4 shows midfrontal cortex from individual with AD (female, aged 83); lane 5 shows cerebellum from the same individual as Lane 4; lane 6 shows normal adult liver (male, aged 18); lane 7 shows normal child lung (male, aged 7).

Figure 6 A depicts self-aggregation of NAC peptide at various times and peptide concentrations (10-300 μM) as measured by turbidity at OD 400 nm. The NAC aggregation was studied in PBS (pH 7.4) solution at 37°C.

Figure 6 B depicts self-aggregation of NAC peptide (concentration, 300 μM) at various times and temperatures (4°, 22°, and 37°C) as measured by turbidity at OD 400 nm. The NAC aggregation was studied in PBS (pH 7.4) solution.

Figure 7 is a Western blot analysis of NAC peptide aggregation. A NAC peptide monomer migrated at an apparent molecular mass of the 3500 Da. The signal intensity of 3500 Da band was significantly decreased on Days 5 and 7. On the other hand, aggregated NAC peptide was found at the top of the gel. This signal increased to a maximum by Day 3. No intermediate-size bands were observed.

Figures 8A and B show birefringence of Congo red-stained NAC peptide viewed by cross-polarization microscopy. Bright-field (A) and cross-polarized light (B) pictures of NAC peptide preparation stained with Congo red are shown.

Figure 9 is an electron micrograph of aggregated NAC peptide.

Figures 10 A through D show (both macro- and microscopically) immunostaining of NACP in rat brain using anti-NACP(131-140) with streptavidin-biotin-peroxidase (SAB) method. In FIGURE 10 A is a macroscopic image of stained sagittal brain section showing that NACP immunoreactivity was relatively strong in the neocortex, olfactory region, caudoputamen, hippocampus, and cerebellar cortex compared to the thalamus and brain stem. Higher magnification microscopic analysis showed a characteristic granular immunoreactivity throughout the brain. In FIGURE 10 B is a microscopic image of stained cerebellar cortex. In FIGURE 10 C is a microscopic image of stained hippocampal dentate gyrus. In FIGURE 10 D is a microscopic image of stained cerebral cortex.

Figures 11 A through I are photographs obtained by laser scanning confocal microscopy of sections double-labeled for SY38 (mouse monoclonal anti-synaptophysin antibody; labeled with FITC and shown by bright patches in panels A, D and G) and NACP(131-140/SEQ.ID.No.6; shown by bright patches in panels B, E and H). The right hand panels (C, F and I) correspond to the electronically merged image; colocalization of NACP with synaptophysin is indicated by bright patches. Panels A-C are derived from staining of neocortex tissue; panels D-F are derived from staining of glomeruli of the olfactory bulb; and, panels G-I are derived from staining of the cerebellar cortex. The scale bar (a horizontal white line across the lower right hand corner of panel A) is equal to 15 μm.

Figure 12 is a bar graph developed by computer-aided quantification of colocalization of NACP with synaptophysin in the presynaptic terminals of rat brain tissue. In the cortical regions a large percentage of the synpatophysin-immunoreactive terminals contained NACP. In contrast, in subcortical regions, a lower proportion of the synapatophysin-immunoalabeled axosomatic nerve terminals contained NACP.

Figure 13 is a photograph obtained by immunoelectron microscopy which reveals the synaptic vesicle membrane localization of NACP. Vibratome sections were immunostained with anti-NACP(131-140) and analyzed by electron microscopy.

Figure 14 is a Western blot quantifying NACP, APP, and synaptophysin in rat brain sections. Immunostaining of NACP, APP, and synaptophysin bands was carried out using anti-NACP(131-140), mouse monoclonal antibody, 22C11, and mouse monoclonal antibody, SY38, respectively. Signal intensity was quantified by scanning by a densitometer. Each value is shown as relative amount of protein normalized to the value in frontal cortex. NACP is highly concentrated in olfactory bulb, frontal cortex, striatum, and hippocampus , whereas APP and synaptophysin are distributed uniformly throughout the brain.

Figure 15 A-B shows, in bar graph form, the number of NACP containing and synaptophysin containing synaptic terminals present per 100 sq/μm of human frontal cortex brain tissue.

Figure 15 C-D shows, in bar graph form, the pixel intensity detected per synapse of human frontal cortex brain tissue indicative of the average quantities of NACP and synaptophysin contained in each synapse. The solid bars are indicative of the values obtained in brain tissue from persons without AD; the slashed bars are indicative of the values obtained in brain tissue from persons suffering from AD.

Figure 16 shows immunolabeling of NACP and synaptophysin in human brain tissue obtained from a healthy person (upper panels) and from a person suffering from AD (middle panels). The left and right images from the middle panel are electronically merged in the lower panel. Areas showing the brightest in the lower panel indicate colocalization of NACP and synaptophysin.

Figure 17 shows immunolabeling of NACP and synaptophysin in mature plaques (the 2 upper left hand panels) and in diffuse plaques (the 2 upper right hand panels). The left and right images from the upper panels are electronically merged in the lower panel. Areas showing the brightest in the lower panel indicate colocalization of NACP and synaptophysin.

Figure 18 shows immunolabeling of β-amyloid (left hand panels) and NAC (right hand panels, with results electronically superimposed on the left hand panels) in brain tissue from healthy, elderly persons (panels A and B), from persons suffering from the early stages of AD (panels C and D), and from persons suffering from advanced stages of AD.

DETAILED DESCRIPTION OF THE INVENTION

A. FUNCTIONAL AND STRUCTURAL CHARACTERISTICS OF NAC AND NACP.

The present invention provides a novel amyloid component (NAC). As used herein "NAC" shall mean Non-Aβ component of AD amyloid. "Aβ" as used herein shall mean fibrillar deposits of the A4 protein, a 39/43 residue amyloid. "AD" as used herein shall mean Alzheimer's Disease. "NAC associated amyloid disorder" shall refer to diseases associated with the excessive formation of amyloid in brain tissue, concommitant synaptic loss, and related cognitive dysfunction. This novel component of amyloid was discovered by analysis of the entire amino-acid sequences in an amyloid preparation of the frontal cortex of patients with typical neuropathological features of Alzheimer's disease (AD) using methods of purification in SDS and sequencing well known in the art. Hence, NAC is the second intrinsic component after Aβ to be found in AD amyloid.

NAC, which is expressed as a larger precursor polypeptide NACP, was found by both biochemical and immunohistochemical evidence to be an intrinsic component of amyloid in AD brain tissue. Copurification of NAC with amyloid in the presence of SDS and immunological localization on amyloid fibrils at the electron microscopic level shows that NAC is localized in neuritic plaques and amyloid fibrils.

As shown in FIGURE 2, NACP is encoded by a 1560 base pair polynucleotide (SEQ. I.D. NO. 1) with a 420 bp open reading frame which encodes a 140 amino acid polypeptide NACP (SEQ. I.D. NO. 2) that is the precursor of NAC, a polypeptide of at least 35 amino acids in length (SEQ. I.D. NO. 3). *In vivo,* NACP is recovered in the cytosolic fraction of human brain homogenate as a protein with an apparent molecular mass of 19,000 Da. NACP has seven repeated KTKEGV amino acid motifs, but no signal petide sequence nor N-linked glyclosylation sites. NAC is located in the most hydrophobic portion of NACP. NAC is at least 35 amino acids and has a molecular weight of approximately Mr 3,500. Within NAC, two new amyloid sequences have been identified and known herein as the "X and Y peptides", which are encoded contiguously in the most hydrophobic domain (SEQ. I.D. NOS. 4 and 5, respectively). The definite length of NAC was not determined due to the use of enzymatic digestion in its preparation.

The association of NAC in amyloid deposits in AD brain tissue differs from that of both Aβ and α1-antichimotrypsin (ATC), two proteins generally used as indicators of the presence of amyloid in Alzheimer's Disease. Recent work has shown that 50% of intracellular neurofibrillary tangles (NFT) and 100% of extracellular NFT contain Aβ (G. Perry, *et al.*, *Am. J. Pathol.*, 140:283-290, 1992). Immunohistochemical studies of the distribution of NAC in AD brain tissue (See Example 2 herein) found that NAC was not present in NFTs.

The association of NAC with amyloid in the brains of patients with the symptoms of Alzheimer's Disease (AD) is high. Although ATC has been reported to be localized on amyloid fibrils in brain tissue at the electron microscopic level (C. R. Abraham, *et al.*, *Cell*, 52:487-501, 1988), biochemical analysis of amyloid AD brain tissue prepared and analyzed as in Examples 1-3 below, revealed no ATC. This result suggests that the amount of ATC in amyloid may be too small to detect in the present preparation, or, alternatively, that the association of ATC with amyloid may be less significant than that of NAC and, therefore, ATC may be lost during preparation of amyloid used herein.

Thus, as an indicator of the deposition of amyloid in brain tissue, NAC is both more specific to neuritic plaque and amyloid fibrils than ATC and less likely to be lost in preparation of tissues to be tested than ATC.

NAC is strongly hydrophobic and has characteristics associated with a tendency to form a β-pleated secondary protein structure. When synthesized chemically, NAC aggregates and precipitates easily in aqueous solution in a time, concentration and temperature-dependent manner. More particularly, synthetic NAC was detected initially as a monomer of 3500 Da, but became aggregated in aqeuous solution into a higher molecular weight molecule that could not migrate into an electrophoretic gel. On Congo red staining, the NAC aggregate showed green-gold birefringence when viewed with a poloarized light microscope and had a fiber-like structure when viewed through an electron microscope.

Based on the relative yield of peptides X, Y and Aβ sequences in amyloid preparations, the concentration of NAC in amyloid seems to be less than ten percent that of Aβ. Further, double-immunostaining of NAC with β-amyloid antibodies revealed that NAC is more abundant in mature than in diffuse plaques. Interestingly, diffuse plaques from "normal" control tissue do not react with anti-NAC, whereas early and advanced AD cases cotaining large numbers of diffuse and/or at least some mature plaques display relatively strong anti-

NAC reactivity (in approximately 30-50% of the plaques) (see, FIGURE 18). These data suggest that (1) there is a connection between metabolism of presynatpic proteins and plaque formation, and (2) NAC follows diffuse β-amyloid accumulation into mature plaques.

In contrast, it does not appear that NACP is substantially present, if at all, in amyloid. For example, no other sequences of NACP besides the X and Y peptides were detected in the peaks eluted from HPLC analysis of the NACP protein. Further, while NAC was identifed in immunostained amyloid on Western or dot blot, NACP was not. Thus, it appears that NAC can form amyloid *in vivo* after cleavage from its precursor (NACP) and is likely to play a substantial role in amyloidosis. However, because the amyloid found in the brain tissue of humans with confirmed diagnoses of AD differs in structure from NAC aggregates alone, it is likely that NAC is not the sole component of amyloid. Rather, it is most probable that NAC is involved in the initial stages of amyloid formation, leaving the principal development of amyloidosis to the accumulation of β-amyloid.

It should be appreciated, however, that observations have been made that proteins that bind to β-amyloid retard its accumulation (see, e.g., Strittmatter, *et al., Proc.Natl.Acad.Sci. USA*, 90:8098-8102 (1993); Fraser, *et al., J.Neurochem.*, 61:298-305 (1993); and, Schwarzman, *et al., Ann.N.Y.Acad.Sci.*, 6:139-143 (1993)). Thus, with the knowledge of NAC's role in amyloidosis set forth herein, it can be reasonably expected that binding of NAC by NAC polypeptides will retard its accumulation as well, thereby slowing the progression of disease associated with amyloid plaque formation.

With respect to NACP, immunostaining indicates that it, like the APP precursor of β-amyloid, is a presynaptic protein. Specifically, using the streptavidin-biotin-peroxidase staining method, rat brain sections were immunostained by an antibody raised to the NACP amino acid sequence from position 131-140 (see, SEQ.ID.No.2) and position 1-9 (*id.*). Throughout the various cortical and subcortical areas of the rat brain, anti-NACP (131-140) and (1-9) immunostained the neuropil in a characteristic punctate pattern. Neuronal cell bodies, glial cells and blood vessels were not immunostained.

NACP does not have a signal sequence, which suggests that NACP proteins remain localized in neuronal cytoplasm where NACP is expressed. However, it has been discovered that NAC can seep out of cells under certain conditions, such as serum deprivation. Further, as shown in FIGURE 4A, the NACP protein is characterized by repetitive motifs. The KTKEGV motif is repeated seven times, but the amino acid positions 2 to 6 are sometimes substituted. In addition, as shown in FIGURE 4B, amino acids 48-56 and 70-78 of the NACP protein are homologous. These repeated motifs can prove useful in determining the secondary and tertiary protein structure as well as the biological function and metabolism of this protein.

For instance, in accordance with the teachings of P. J. Kennelly, *et al.* (*J. Biol. Chem.*, 256:15555-15558, 1991), the threonine residues in the KTKEGV motif would offer favorable targets for protein kinase C (PKC). The action of this enzyme is known to be critical in determining the functional state of neurons (Y. Nishizuka, *Nature*, 334:661-665, 1988).

In addition, laser scanning confocal microscopic analysis of sections double immunolabeled with antibodies against NACP and synaptophysin (a synaptic vesicle protein; see, e.g., Masliah, *et al., J.Neurosci.*, 11:2759-2767 (1991)) showed that both markers colocalized in the great majority of the presynaptic terminals, and that NACP is colocalized with synaptophysin in approximately 80% of the presynaptic boutons and in the neuritic component of plaque (FIGURES 11 through 17). Ultrastructural analysis of sections immunolabeled with NACP confirmed the synpatic localization of this protien and showned that NACP was associated with the synaptic vesicles (FIGURE 13). As compared to synaptophysin and APP (which are distributed fairly evenly throughout the brain), NACP was concentrated in the telecephalon, suggesting a functional role for NACP in that region of the brain (FIGURE 14).

As shown in FIGURE 3, NACP is detected in the cytosolic fraction of brain homogenates and comigrates on Western blots with NACP synthesized in *E. coli* from NACP cDNA. NACP was not detected in a particulate fraction from human cortex or from NACP-expressing *E. coli*. NACP mRNA is expressed principally in the brain, but is also expressed in low concentration in all tissues examined except in liver, suggesting that it has ubiquitous functions as well as brain specific functions.

Interestingly, in AD brain, the total population of NACP-containing presynaptic terminals is significantly diminished (by 30-40% see, FIGURE 15) as compared to "normal" brain tissue; i.e., brain tissue without a diagnostically significant quantity of plaque (defined further below). At the same time, although the total population of such terminals is decreased in the AD brain, the concentration of NACP in each remaining presynaptic bouton, indicating a mechanism to compensate for the overall level of NACP-expressing terminals.

As further evidence of NACP as a synaptic protein, search of the EMBL/GenBank DNA sequence databases reveals homologies between NACP and rat brain synucleins, the electric organ synpase of *Torpedo californica* (Pacific electric ray) and to bovine phosphoneuroprotein 14, a brain specific protein present in synapses around neurons but not in glial cells and Purkinje cell bodies. This group of small, acidic, brain-specific proteins have common repetitive sequence motifs and similar hydrophobic profiles (see, Maroteaux, *et al., Mol.Brain Res.,* 11:335-343 (1991); Maroteaux, *et al., J.Neurosci.*, 8:2804-2815 (1988); and, Nakajo, *et al., Eur.J.Biochem.*, 217:1057-1063 (1993)).

In addition, according to the GenBank database, homology exists between NACP and EST01420, a human 223 bp sequence recently identified by random sequencing of human brain cDNA (M. D. Adams, *et al., Nature*, 355:632-634, 1992). Comparison of the sequence of these proteins expressed in the human brain, as shown in FIGURE 4C, indicates the two proteins are substantially homologous in the N-terminal region, but the EST01420 sequence has the termination codon at base pair position 206, and, therefore, could encode only 51 amino acids.

NACP therefore appears to be a member of a family of synaptic proteins having hydrophobic regions centered in an otherwise hydrophilic molecule.

B. NAC/NACP POLYNUCLEOTIDES AND POLYPEPTIDES.

The term "substantially pure" means any NAC or NACP polypeptide of the present invention, or any gene encoding a NAC or NACP polypeptide, which is essentially free of other polypeptides or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature.

As used herein, the term "functional polypeptide" refers to a polypeptide which possesses a biological function or activity that is identified through a defined functional assay and which is associated with a particular biologic, morphologic or phenotypic alteration in the cell. The biological function can vary from a polypeptide fragment as small as an epitope to which an antibody molecule can bind to as large as a polypeptide which is capable of participating in the characteristic induction or programming of phenotypic changes within a cell. A "functional polynucleotide" denotes a polynucleotide which encodes a functional polypeptide as described herein.

For example, preferred NAC/NACP polypeptides of the invention will be those which will effectively cross the blood/brain barrier without toxic effect. NAC polypeptides of the invention will specifically bind NAC *in vivo*; the peptides will, therefore, have at least one binding site for NAC.

Further, the NAC/NACP polypeptides should not be pathogenic or immunogenic. To the former end, the peptides are soluble and, in the case of NAC peptides, will reversibly bind NAC. To the latter end, the polypeptides are preferably purified from a human or will be synthesized. "Synthesized" in this context refers to peptides produced through human intervention, whether by chemical synthesis, recombinant genetic techniques or modification of an isolated native peptide.

It should be noted that NAC/NACP polypeptides used in the method of the invention may differ in amino acid sequence or structure but still retain the same biological activity as described above. Such modifications may be deliberately made (by, for example, site-directed mutagenesis) or may occur spontaneously. In either case, the invention will encompass the use of NAC/NACP peptides which have the same phenotype regardless of differences in structure and length between the peptides. These phenotypically similar peptides will be considered to "substantially similar" to one another.

On the molecular level, a molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Minor modifications of the NAC primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the NAC polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of NAC still exists. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which may not be required for NAC biological activity.

The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

By "functional derivative" is meant the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the DNA sequences of the present invention, includes any nucleotide subset of the molecule. A "variant" of such molecule refers to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

Similarly, a "functional derivative" of a gene encoding NACP polypeptide of the present invention includes "fragments", "variants", or "analogues" of the gene, including degenerate polynucleotides whose sequence may be determined readily by one of ordinary skill in the art, which encode a molecule possessing similar activity to a NAC peptide or fragment thereof.

Thus, as used herein, NAC or NACP polypeptide and NAC or NACP polynucleotide, include any functional derivative, fragments, variants, analogues, chemical derivatives which may be substantially similar to the NAC polypeptides and polynucleotides described herein and which possess similar activity.

Peptides of the invention can be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, 85:2149, (1962), and Stewart and Young, *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

As used herein, the terms "polynucleotide" or "NACP polynucleotide" denotes DNA, cDNA and RNA which encode NACP polypeptide as well as untranslated sequences which flank the structural gene encoding NACP. It is understood that all polynucleotides encoding all or a portion of NACP polypeptide of the invention, such as the NAC polypeptide(s) are also included herein, as long as the encoded polypeptide exhibits the activity or function of NACP or the tissue expression pattern characteristic of NACP. Such polynucleotides include naturally occurring forms, such as allelic variants, and intentionally manipulated forms, for example, mutagenized polynucleotides, as well as artificially synthesized polynucleotides. Such mutagenized polynucleotides can be produced, for example, by subjecting NAC or NACP polynucleotide to site-directed mutagenesis.

As described above, in another embodiment, a polynucleotide of the invention also includes in addition to NACP and/or NAC coding regions, those nucleotides which flank the coding region of the NACP structural gene. For example, a polynucleotide of the invention includes 5' regulatory nucleotide sequences and 3' untranslated sequences associated with the NACP structural gene.

The polynucleotide sequence for NACP also includes antisense sequences. The polynucleotides of the invention also include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, as long as the amino acid sequence of NACP results in a functional polypeptide (at least, in the case of the sense polynucleotide strand), all degenerate nucleotide sequences are included in the invention. Where the antisense polynucleotide is concerned, the invention embraces all antisense polynucleotides capable of inhibiting production of NACP polypeptide.

The preferred NACP cDNA clone of the invention is defined by a sequence of 1560 basepairs, in accordance with the transcript of 1.6 kb. A minor transcript of 3.6 kb is also found. The sequence surrounding the predicted initiator methionine codon (GCCATGG) agrees with the Kozak consensus sequence according to K. Kozak (*Nucleic Acids Res.*, 15:8125-8148, 1987). The nearest in-frame stop codon is found 18 bp upstream of the ATG initiation codon. As shown in FIGURE 2A, the nucleotide sequences encoding the X- and Y-peptide sequences are localized contiguously in the middle of the precursor peptide at bp 233 to 337. The preferred NACP cDNA clone is characterized by the lack of a sequence encoding a signal peptide and by the lack of N-linked glycosylation sites.

DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to : 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes where each probe is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucleic Acid Research*, 9:879, 1981).

A NACP containing cDNA library can be screened by injecting the various mRNA derived from cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for NACP or the X or Y peptide fragments thereof polypeptide or by using probes for the repeat motifs and a tissue expression pattern characteristic of NACP. Alternatively, a cDNA library can be screened indirectly for NACP polypeptides having at least one epitope using antibodies specific for the polypeptides, such as X and Y peptides. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of NACP cDNA.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically.

This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA.

For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucl. Acid Res.*, 9:879, 1981).

The development of specific DNA sequences encoding NACP, or fragments thereof, can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of these three methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al., Nucl. Acid Res.*, 11:2325, 1983).

A cDNA expression library, such as lambda gt11, can be screened indirectly for NACP or NAC peptides having at least one epitope, using antibodies specific for NACP or the NAC peptide. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of NACP cDNA.

DNA sequences encoding NACP or NAC can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, in other words when the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the NACP or NAC polynucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the NACP genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al., Gene*, 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.*, 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters and enhancer).

Polynucleotide sequences encoding NACP or NAC peptides can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the NACP or NAC proteins of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Isolation and purification of microbially expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

One skilled in the art will also be able to create a living mouse model for expressing the NACP gene in a living transgenic mouse. Methods of making a transgenic mouse expressing a foreign gene utilize several techniques for inserting the foreign gene into the germline of the animal at an early developmental stage, such as at the single-cell level. For instance, the transgene can be inserted into a mouse oocyte, which is then implanted into mouse for birth of a transgenic animal. See U. S. Patent No. 4,873,191, "Genetic Transformaion of Zygotes," which is incorporated herein in its entirety. Similarly, pluripotent embroyo-derived stem (ES) cells, can be modified extra-corporeally by insertion of a cloned gene to transfer a modification to the germ line of a living organism.

Homologous recombination has also been used for targeting genetic mutations to a predetermined genetic locus of an ES cell in order to produce a transgenic animal (Mansour, *et al.*, *Nature*, 336:348, 1988; Capecchi, M., *Trends Genet.*, 5:70, 1989). Homologous recombination between DNA sequences residing in the chromosome and newly introduced cloned DNA sequences allows the transfer of any modification to the cloned gene into the genome of a living cell. Several site-specific recombination systems are known (Craig, *Ann. Rev. Genet.*, 22:77, 1988) including the FLP system of yeast and the Cre system of bacteriophage P1. The FLP recombinase of the yeast *saccharomyces cerevisiae* acts on copies of a recombination target called FRTs. The FLP system has been shown to effect site-specific recombination in the *Drosophilia* genome *in vivo* (Golic, *et al., Cell*, 59:499, 1989, Golic, K., *Science*, 252:958, 1991) and in eukaryotic cells *in vitro* (O'Gorman, *et al., Science*, 251:1351, 1991).

A novel approach to effecting specific homologous recombination events in eukaryotes is the prokaryotic Cre-loxP site-specific DNA recombination system of coliphage P1. The 38 kD Cre protein efficiently causes both inter- and intramolecular recombination between specific 34 base pair repeats termed loxP (Stemberg, *et al., J. Mol. Biol.*, 150:467, 1981). Each loxP site contains two 13 base pair inverted repeats and an 8 base pair asymmetric core sequence. No accessory proteins are required for exchange to occur. Direct repeats of loxP dictate an excision of intervening sequences while inverted repeats specify inversion. Cre has been shown to be functional in eukaryotic cells (Sauer, *et al., Nucleic Acids Res.*, 27:147, 1989) and in transgenic plants (Dale, *et al., Proc. Natl. Acad. Sci. USA*, 88:10558, 1991).

These and other types of "gene targeting" provide a means for controlling the site of integration (Smithies, *et al., Nature*, 317:230, 1985). For homologous recombination to occur between two DNA molecules, the molecules must possess a region of sequence identity with respect to one another, typically several hundred base pairs in length. This method requires that the gene of interest must have been previously cloned, and the intron-exon boundaries determined, as is the case herein. Targeted insertion increases the probability that an inserted gene will function as desired. It also reduces the chance of random insertion activating a quiescent oncogene or inactivating a cancer supressor gene.

U. S. Patent Nos. 5,175,383, 5,175,381 and 5,175,385, which are incorporated herein by reference in their entirety, illustrate utilization of these methods of targeted insertion to either correct or mutate a desired chromosomal locus, thereby creating a transgenic animal. U. S. Patent No. 5,175,383 discloses the method of making a transgenic mouse model for the human disease benign prostatic hypertrophy. To create the Harvard mouse the int-2 oncogene, which codes for a growth factor, was joined to a control gene to ensure that the growth factor would be produced in prostate tissue. Systems for studying regulation of genes in transgenic animal are also disclosed in Patent Application WO 90/06367 entitled "Transgenic Mice for the Analysis of Hair Growth" wherein insertion of a promoter of a gene for a hair specific protein, one expressed only in tissues involved in hair growth, is used to regulate expression of a reporter gene. Due to conservation among homologous genes and their products, transgenes can be expressed in mice under the control of a regulatory sequence from a human tissue specific gene. Recently, Patent Application WO 93/14200, which is incorporated herein by reference in its entirety, discloses creation of a trangenic mouse that expresses β-amyloid precursor proteins.

D. METHODS FOR USE OF NAC ANTISENSE POLYNUCLEOTIDES

The NAC polynucleotide in the form of an antisense polynucleotide is useful in treating disease states associated with formation of amyloid i.e., amyloidosis in the brain, (particularly in neuritic) plaques by preventing expression of the protein that is originating. Essentially, any disorder which is etiologically linked to expression of NACP could be considered susceptible to treatment with a reagent of the invention which modulates NACP expression. The term "modulate" envisions the suppression of expression of NACP when it is over-expressed, or augmentation of NACP expression when it is under-expressed or when the NACP expressed is a mutant form of the polypeptide.

When amyloidosis is associated with NACP overexpression, such suppressive reagents as antisense NACP polynucleotide sequence or NACP binding antibody can be introduced to a cell. Alternatively, when an amyloid disorder is associated with underexpression or expression of a mutant NACP polypeptide, a sense polynucleotide sequence (the DNA coding strand) or NACP polypeptide can be introduced into the cell. Methods for use of antisense gene therapy are discussed in greater detail below.

E. ANTI-NAC AND ANTI-NACP ANTIBODIES.

The invention includes polyclonal and monoclonal antibodies immunoreactive with NACP or NAC polypeptides or immunogenic fragments thereof.

Antibodies which are specific for NAC or NACP may be produced by immunization of a non-human with antigenic NAC or NACP peptides of native or synthetic origin. Once antigenic peptides are prepared, antibodies to the immunizing peptide are produced by introducing peptide into a mammal (such as a rabbit, mouse or rat).

A multiple injection immunization protocol is preferred for use in immunizing animals with the antigenic MTA peptides (see, e.g., Langone, *et al.*, eds., "Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections", *Methods of Enzymology* (Acad. Press, 1981). For example, a good antibody response can be obtained in rabbits by intradermal injection of 1 mg of the antigenic MTA peptide emulsified in Complete Freund's Adjuvant followed several weeks later by one or more boosts of the same antigen in Incomplete Freund's Adjuvant.

If desired, the immunizing peptide may be coupled to a carrier protein by conjugation using techniques which are well-known in the art. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g. a mouse or a rabbit).

Polyclonal antibodies produced by the animals can be further purified, for example, by binding to and elution from a matrix to which the peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (see, for example, Coligan, *et al.*, Unit 9, *Current Protocols in Immunology*, Wiley Interscience, 1991).

If desired, polyclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which NAC polypeptide is bound. Those of skill in the art will know of various other techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies.

Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art (Kohler, *et al., Nature,* 256:495, 1975). The term antibody or, immunoglobulin as used in this invention includes intact molecules as well as genetically engineered antibody constructs such as bifunctional antibodies, CDR grafted antibodies, and the like, as well as fragments thereof, such as Fab and F(ab')$_2$, which are capable of binding an epitopic determinant on NACP or NAC.

A preferred method for the identification and isolation of an antibody binding domain that exhibits binding with NACP or NAC peptides is the bacteriophage λ vector system. This vector system has been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in *Escherichia coli* (Huse, *et al., Science,* 246:1275-1281, 1989) and from the human antibody repertoire (Mullinax, *et al., Proc. Natl. Acad. Sci.,* 87:8095-8099, 1990). As described therein, receptors (Fab molecules) exhibiting binding for a preselected ligand were identified and isolated from these antibody expression libraries. This methodology can also be applied to hybridoma cell lines expressing monoclonal antibodies with binding for a preselected ligand. Hybridomas which secrete a desired monoclonal antibody can be produced in various ways using techniques well understood by those having ordinary skill in the art and will not be repeated here. Details of these techniques are described in such references as *Monoclonal Antibodies-Hybridomas: A New Dimension in Biological Analysis,* Edited by Roger H. Kennett, *et al.*, Plenum Press, 1980; and, U.S. Patent No. 4,172,124.

F. METHODS FOR DETECTING AMYLOID OR A CELL EXPRESSING NACP.

The invention provides a method for detecting a cell expressing NACP, or an amyloid disorder associated with NAC, comprising contacting a cell suspected of expressing NACP or having a NAC associated disorder with a reagent which binds to the target component. The cell component can be nucleic acid, such as DNA or RNA, or protein. When the component is nucleic acid, the reagent is a nucleic acid probe or PCR primer. When the cell component is protein, the reagent is an antibody probe. The probes are detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody or probe, or will be able to ascertain such, using routine experimentation.

For purposes of the invention, an antibody or nucleic acid probe specific for NACP or fragments thereof may be used to detect the presence of NACP polypeptide or NAC peptides (using antibody) or polynucleotide (using nucleic acid probe) in biological fluids or tissues. Any specimen containing a detectable amount of antigen can be used. A preferred sample in this invention is tissue of brain origin, specifically midfrontal cortex tissue obtained through biopsy. More preferably, the tissue is hippocampus tissue. Preferably the subject is human.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies.

The method for detecting a cell expressing NACP or a amyloid disorder associated with NAC, described above, can be utilized for prescreening for detection of amyloidosis prior to or after a subject's manifestation of typical clinical and neuropathological features of AD. Additionally, the method for detecting NACP polypeptide in cells is useful for prescreening to detect risk of amyloid disorder by identifying cells expressing NACP at levels different than normal cells. Using the method of the invention, high, low, and mutant NACP expression can be identified in a cell and the appropriate course of treatment can be employed (e.g., sense or antisense gene therapy).

The monoclonal antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of NACP or NAC peptides such as X and Y. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

As used in this invention, the term "epitope" includes any determinant capable of specific interaction with the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The concentration of detectably labeled monoclonal antibody which is administered should be sufficient such that the binding to those cells having NAC or NACP is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

Because of the blood/brain barrier, it can be expected that antibodies will not be particularly the preferred reagant for use in *in vivo* applications. Rather, NAC/NACP polypeptides (particularly the former) that will cross the blood-brain barrier, and bind to the native protein are expected to be the best NAC/NACP ligands. In particular, the preferred ligands of the invention will be those which are derived from positions 61-95 of the NACP amino acid sequence (i.e., in the NAC region), and from the C-terminal region of NACP (positions 131-140). In part, this preference is derived from the observation that antibodies to NAC (61-95) bind amyloid plaques, while antibodies to NACP (131-140) bind synapses, as well as the presumed ability of the peptides to cross the blood brain burner.

It has been shown that β-amyloid peptide fragments of about 28 amino acids in length or shorter will effectively cross the blood/brain barrier *in vivo* without toxic effect (see, e.g., Examples 15 and 16, as well as co-pending, commonly owned U.S. Patent Application No. 08/136,751; filed 10/14/93). Given the similarity in structure between the pleated β-amyloid molecule and NAC (see, Example 8, below), it can be expected that NAC peptides of about 28 amino acids or shorter in length would cross the blood-brain barrier. As shown in Example 8, NAC is a self-aggregating peptide (which apparently derives from the 61-95 amino acid region of NACP). Within the 61-95 stretch of amino acids (see, SEQ.ID.No.1), the following peptides have been determined to have self-aggregating ability (i.e., binding sites for NAC) using the method described in Example 8 (reading from the N to the C terminus):

TVEGAGSIAAATGFVKKD (NAC peptide 1)
and KKKTVEGAGSIAAATGFV (NAC peptide 2).

Further, the somewhat shorter NACP peptides described below (which are derived from the 131-140 region of NACP; see, SEQ.ID.No.1), would also be expected to cross the blood/brain barrier (reading from the N to the C terminus):

EGYQDYEPEAKKD (NACP peptide 1)
and KKKEGYQDYEPEA (NACP peptide 2).

Determination of whether a particular NAC or NACP peptide will specifically bind NAC or NACP can be readily made without undue experimentation by one of ordinary skill in the art. In regard to characterstics of peptides that may cross the blood/brain barrier, those of skill in the art may wish to refer to Pardridge, WA, "*Peptide Drug Delivery to the Brain*", (Raven Press, 1991), chapters 3, 6 and 7 of which in particular are incorporated herein by this reference to illustrate the state of knowledge in the art concerning delivery of peptides to the brain. An example of a suitable animal model and testing protocol for use in this regard are set forth in Examples 15-16.

As an illustration of techniques which may be employed to identify peptides that may cross the blood/brain barrier, without undue experimentation, peptides shorter than NAC (61-95) or NACP (131-140) can be screened for use in the method of the invention by incubation with AD brain tissue homogenates or brain tissue from an animal model which has been implanted surgically with amyloid, or through immunological techniques such as those described above (e.g., testing the reactivity of anti-NACP antibodies that react with the native protein to the candidate ligand).

It is also possible to determine without undue experimentation if a NAC or NACP peptide (i.e., NAC or NACP candidate ligand) has the same specificity as the NAC ligand described above by ascertaining whether the former prevents the latter from binding to NAC. If the candidate ligand competes with a ligand which is known to bind NAC/NACP (as shown by a decrease in binding by the latter), then the two peptides bind to the same, or a closely related site.

Still another way to determine whether a particular candidate ligand has the specificity of a ligand which is known to bind NAC/NACP is to preincubate the candidate ligand with NAC or NACP and then add a known ligand to determine if it is inhibited in its ability to bind NAC or NACP. If the known NAC/NACP ligand is inhibited, in all likelihood the candidate ligand has the same, or functionally equivalent, binding specificity as the known NAC/NACP ligand.

Screening of candidate ligands can also be determined by attaching a detectable label to them, incubating them with amyloid-containing brain tissue (*in vivo* or *in vitro*) and determining whether binding has occurred using *in vivo* diagnostic imaging techniques as described in more detail below.

NAC and NACP ligands may be labelled as described below; however, for *in vivo* diagnostic imaging, the use of radiolabels or paramagnetic isotopes will be preferred. For example, for *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to proteinaceous ligands either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the ligands of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl. However, for its relatively low toxicity and ready imaging, [TM^{99}] (pertechenetate) will be the most preferred radiolabel for its relatively low toxicity in mammals. Radiolabelling with ^{99m}Tc may be performed according to the technique described in Kasnia, *et al. J. Nucl. Med.*, 32:1445-1451, 1991.

However, for any *in vitro use,* ^{125}Iodide (^{125}I) would be preferred for ease of detection. ^{125}I may be attached to a NAC or NACP ligand for use in the invention by conventional techniques including oxidative radiodination using sodium ^{125}I and chloramine T (for tyrosine containing peptides) or the acylation followed by oxidative radiodination (for peptides not containing tyrosine). Iodination may also be performed using an iodination product from DuPont of Wilmington, DE (marketed under the trademark NEN) or the iodogen technique described in Salacinski, *et al., Anal. Biochem.*, 117:136-146, 1981. Iodogen for use in this method is commercially available from Pierce and Wariner, Chester, England.

The ligands of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Regardless of the detectable label used, the labelled ligands will preferably be purified by means well known in the art referred to above (for example, RP-HPLC) to an essentially quantitative specific activity (e.g., about 2000 Ci/mmol; 1Ci≈ 37GBq).

To practice the invention, a diagnostically effective amount of a detectably labelled ligand as described above will be administered to mammal which is suspected of having AD, has been diagnosed as having AD or, in the research context, has had amyloid plaque formation induced in its brain tissue. In the preferred embodiment, the mammal will be a human who is suspected of having or has been diagnosed as having AD.

As a rule, the dosage of detectably labeled ligand for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about 0.001 mg/m^2 to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, amyloid burden, and other factors known to those of skill in the art.

Those skilled in the art will be able to determine an appropriate dosage for the detectably labelled ligands based on the animal study data provided in the examples below. In general, the "diagnostically effective amount" of detectably labelled NAC ligand for *in vivo* applicants will be that amount which is sufficient to detectibly bind any NAC present in the subject brain tissue.

Although binding of the detectably labelled peptides of the invention is somewhat dose-dependent, it will be appreciated because the peptides are self-aggregating, increasing their dosage may intensify rather than expand the NAC binding pattern. More specifically, while binding of most or all of the NAC plaques present in the subject brain tissue may occur at lower dosage levels, the intensity of the emissions indicative of that binding may be enhanced as the detectably labelled peptide density per plaque is increased at higher dosage levels.

Although any nonsurgical route of administration which introduces the detectably labelled ligands of the invention into brain tissue may be used, intraarterial injection in preferred, with intracarotid injections being most preferred. Where the method of the invention is being performed for diagnostic purposes, the background measurement will preferably be established by determining the extent of binding of a detectably labelled ligand in healthy mammalian subjects. In this context, "healthy" is defined as a mammal with less than about 15 amyloid plaques/unit area of brain tissue (one unit area = 0.1 square millimeter) and/or a subject who exhibits no clinical signs of a neuropsychological disorder. In the same regard, measurements based on binding of the detectably labelled ligand indicative of the presence of ≥ about 15 plaques/unit area will be considered to be diagnostically significant for (i.e., indicative of) AD. These data can be used to assist in confirmation or refutation of a clinical diagnosis of AD.

To evaluate the prognosis of a subject who is suspected of having or has been diagnosed as having AD, the method of the invention can provide data of at least three significant types. First, using NAC ligand, plaque density in excess of about 15 plaques/unit area can be correlated to the progress of the disease (i.e., greater plaque density would be expected to be present in later stages of the disease). Second, measurements of plaque development taken over time will be indicative of the rate at which the disease is progressing and/or of the effectiveness of a particular treatment or therapy. In applying the inventive method to evaluate the progression of the disease, previous plaque density measurements taken from that subject would be used as background. Third, using NACP ligand, the probable progression of loss of cognitive function as well as the status of plaque formation through NACP cleavage, may be evaluated based on decreses in the synaptic population and/or increases in presynatpic bouton concentrations of NACP.

Binding will preferably be measured within one hour of introducing the detectably labelled peptide into the subject's bloodstream. Binding will be measured *in vivo* using well-known *in vivo* diagnostic imaging techniques (in particular computer assisted sectional radiography (tomography)), preferably during the first hour following administration of the detectably labelled peptide.

Of the presently known tomography techniques, positron emission tomography (PET) and single photon emission computed tomography (SPECT) are preferred for use in the method of the invention. Because the appropriate use of these techniques will be known or apparent to those skilled in the art, their use will not be described in detail here.

For both prognosis and diagnosis, it may be desirable to evaluate the results of the *in vivo* binding assay of the invention in combination with evidence of the synaptic integrity of the subject brain tissue as well as clinical signs of disease. One suitable *in vitro* technique for evaluating and detecting synaptic loss in sections of brain tissue using anti-synaptophysin antibodies is described in

Masliah, *et al., Am. J. Pathol.,* 137:1293-1297 (1990), the disclosure of which is hereby incorporated by reference to demonstrate techniques for evaluating synaptic loss known in the art; other techniques will be known to those of skill in the neurological arts.

For research purposes, the NAC/NACP ligands may be used for *in vitro* studies, for example, binding affinity of different peptides, to develop anti-amyloid antibodies, to study the pathology of amyloid deposition and to evaluate proposed therapies. The NAC/NACP peptides may be of particular use in developing *in vivo* means of differential diagnosis. For example, because NAC principally appears in mature plaques, the stage of AD development is a given patient may be identified more accurately by detecting NAC *in vivo* than is now possible using conventional diagnostic techniques.

More generally, the NAC/NACP ligands of the invention can be used to monitor the course of amelioration of NAC associated amyloid disorder. Thus, by measuring the increase or decrease in the number of cells expressing NACP or changes in the concentration of normal versus mutant NACP or NAC present in various body fluids and/or tissues, it would be possible to determine whether a particular therapeutic regiment aimed at ameliorating the disorder is effective.

G. THERAPEUTIC METHODS FOR TREATING NAC ASSOCIATED AMYLOID DISORDER.

The present invention also provides a method for treating a subject with a NAC associated amyloid disorder. Because the NACP nucleotide sequence can be expressed in an altered manner as compared to expression in a normal cell, it is possible to design appropriate therapeutic or diagnostic techniques directed to this sequence. Thus, where an amyloid disorder is associated with the over-expression of NACP, nucleic acid sequences that interfere with NACP expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid and ribozymes to block translation of a specific NACP mRNA, either by masking that mRNA with an antisense nucleic acid or by cleaving it with a ribozyme. In cases when a amyloid disorder or abnormal cell phenotype is associated with the under expression of NACP or expression of a mutant NACP polypeptide, nucleic acid sequences encoding NACP (sense) could be administered to the subject with the disorder.

Further, as indicated in the Background of the Invention, studies regarding accumulation of β-amyloid in brain tissue have indicated that binding of synthetic β-amyloid peptide to native β-amyloid actually retards the accumulation of the native protein. Based on these results, and given the physical and functional relationships between NAC and β-amyloid (see, Examples 12 and 13 below), it can be expected that administration of NAC peptides will provide a therapeutic benefit to a person suffering from a NAC associated amyloid disorder, such as AD. NAC peptides that will cross the blood-brain barrier and bind to NAC (to "modulate" the accumulation thereof) are identified elsewhere above, as are means to identify any additional NAC peptides possessing this ability.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, *Scientific American*, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target NACP-producing cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.*, 172:289, 1988).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, *J. Amer. Med. Assn.*, 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, *Nature*, 334:585, 1988) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that that sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

The present invention also provides gene therapy for the treatment of amyloid disorders which are mediated by NACP protein. Such therapy would achieve its therapeutic effect by introduction of the NACP antisense polynucleotide, into target cells (i.e., in brain tissue) of subjects having the amyloid disorder. Delivery of antisense NACP polynucleotide can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Disorders associated with under-expression of NACP could similarly be treated using gene therapy with sense nucleotide sequences.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a NACP sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding an enzyme that determines the structure of a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the NACP antisense polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence that enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include but are not limited to ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag, pol* and *env*, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for NACP antisense polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 um can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to brain cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). For instance *in vivo* administration can be in a bolus or by gradual perfusion over time by means adapted for crossing the blood-brain barrier. For instance, the NAC or NACP polynucleotides or polypeptides can be injected by epidural administration or intralumbar puncture using standard techniques well known to the medical profession, although an intrarterial route of administration would be preferred for patient comfort.

In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

The compounds bound to the surface of the targeted delivery system will generally be ligands and receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest which will bind to another compound; e.g., NAC.

In general, surface membrane proteins which bind to specific effector molecules are referred to as receptors. In the present invention, antibodies are preferred receptors. Antibodies can be used to target liposomes to specific cell-surface ligands. For example, certain antigenic sites associated with NAC polypeptides in amyloid formations may be exploited for the purpose of targeting NAC polypeptide or polynucleotide containing liposomes directly to the amyloid deposit. Since the NACP gene product may be indiscriminate with respect to cell type in its action, a targeted delivery system offers a significant improvement over randomly administered non-specific liposomes. Preferably, the target tissue is brain tissue and the target cell is a neuron/glia.

A number of procedures can be used to covalently attach either polyclonal or monoclonal antibodies to a liposome bilayer. Antibody-targeted liposomes can include monoclonal or polyclonal antibodies or fragments thereof such as Fab, or $F(ab')_2$, as long as they bind efficiently to an antigenic epitope on the target cells. Liposomes may also be targeted to cells expressing receptors for hormones or other serum factors.

H. KITS AND ASSAYS FOR USE IN THE METHODS OF THE INVENTION.

The antibodies and substantially purified NAC peptide of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of the container means comprising the separate elements of the assay to be used.

The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunometric, or sandwich immunoassays.

The term "immunometric assay" or "sandwich immunoassay", includes simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

In performing the assays it may be desirable to include certain "blockers" in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, proteases, or anti-heterophilic immunoglobulins to anti-NACP or anti-NAC immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention.

It has been found that a number of nonrelevant (i.e., nonspecific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g., IgG1, IgG2a, IgM, etc.) can be used as "blockers". The concentration of the "blockers" (normally 1-100 μg/μl) is important, in order to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in the specimen.

I. PHARMACEUTICAL COMPOSITIONS OF THE INVENTION.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the polynucleotides, monoclonal antibodies or the NAC/NACP ligands of the invention ("pharmaceutically active molecules"), the medicament being used for therapy of NAC associated amyloid disorders.

Pharmaceutically active molecules will preferably be administered in a pharmaceutically acceptable carrier, which may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. A pharmaceutically active molecule of the invention may be conjugated by means well known in the art to polyethylene glycol (PEG) to reduce its immunogenicity.

Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

The following examples illustrate the manner in which the invention can be practiced. It is understood, however, that the examples are for the purpose of illustration and the invention is not to be regarded as limited to any of the specific materials or conditions therein.

EXAMPLE 1

AMYLOID PREPARATION

Amyloid was purified from the frontal cortex of patients with typical clinical and neuropathological features of AD (obtained from the Alzheimer's Disease Research Center, San Diego, CA) using a modification of a protocol previously described by J. Kondo, *et al.* (*Neuron*, 1:827-834, 1988). Briefly, AD cortex was homogenized in 2% SDS, 1% 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.6, heated to 95°C for 10 minutes, and then centrifuged at 100,000 x g for 60 minutes. The pellets were suspended in 50 mM Tris-HCl, pH 7.6, and 1% SDS (SDS buffer) and centrifuged at 100,000 x g for 60 minutes. The pellets were resuspended in 0.5 M sucrose in SDS buffer and subjected to 1.0/2.0 M sucrose step-gradient centrifugation at 245,000 x g for 2 hours. The interfaces were collected and centrifuged at 150,000 x g for 60 minutes after 5 times dilution with SDS buffer.

The pellets recovered were sonicated in SDS buffer, dialyzed against 70% formic acid, cleaved with CNBr, and digested with *Achromobacter lyticus* protease I in 5 M urea at 30°C for 5 hours. The cleaved peptides were separated by HPLC on a C4 column with a linear gradient (0-80%) of acetonitrile/isopropanol (3/7) in 0.1% trifluoroacetic acid. All of the HPLC peaks eluted from HPLC were sequenced using methods known in the art. In addition to the major Aβ sequence (31.1 nmol) two heretofore unknown peptides X (2.0 nmol) and Y (2.3 nmol) were recovered. All of the amino acid sequences found could be attributed to proteins known to be associated with amyloid tissue--Aβ protein, τ, ubiquitin, ferritin, and collagen--except for the two heretofore unknown peptides, named herein as NAC peptide X (SEQ. I.D. NO. 4) and NAC peptide Y (SEQ. I.D. NO. 5). No other sequences of NACP other than peptides X and Y were detected in the amyloid preparation.

Because Peptides X and Y were recovered in essentially the same concentration, the hypothesis was drawn that they were derived from a single larger precursor peptide NACP. Because the amyloid fraction contained sequences of τ and ubiquitin, known components of PHF, it was possible that the X and Y peptides might have derived from contaminating PHF. To exclude this possibility, it was necessary to localize X and Y with immunological probes.

EXAMPLE 2

ANTIBODY PRODUCTION AND IMMUNOHISTOCHEMISTRY

Proteins X and Y were synthesized using methods known in the art, tested for solubility, and found not to be soluble in any aqueous solution examined. Therefore, the N-terminal sequences of proteins X and Y were synthesized and used to raise rabbit antisera using methods known in the art. For example, a C-terminal cystein was added to the 9 N-terminal amino acids of protein X to form a protein fragment X1 EQVTNVGGAC (SEQ. I.D. NO. 6), and a C-terminal cystein was added to the 7 N-terminal amino acids of protein Y to form a protein fragment Y (TVEGAGSC) (SEQ. I.D. NO. 7). These protein fragments X1 and Y were conjugated to KLH using MBS as described in N. Green, *et al.*, (*Cell 28*:477-487, 1982). Rabbits were boosted several times with the MBS-conjugated peptides, and then proteins X and Y were conjugated to KLH with glutaraldehyde, injected into rabbits, and antisera were obtained as described in E. Masliah, *et al.* (*J. Neurosci.*, 10:2113-2124, 1990).

Antisera obtained from the rabbits were used to perform immunohistochemical and immunoelectron microscope analysis of AD brain sections using methods known in the art (Masliah, *supra*, 1990; E. Masliah, *et al.* (*J. Neurosci.*, 11:2759-2767, 1991). In particular, specificity of the staining was demonstrated by a pre-absorption experiment consisting of incubation of antibody X1 with peptide X1 and incubation of antibody Y with peptide Y. In the control, the antisera were incubated with peptide of an entirely different sequence (EGYQDYEPEAC) (SEQ. I.D. NO. 8).

In order to define which structures were immunostained (neurofibrillary tangles, neuritic plaques, vascular amyloid, and neuropil threads) and to identify plaque subtypes (diffuse, primitive, and mature), some of the immunostained sections were further stained with a 1% aqueous solution of thioflavin S and were viewed with ultraviolet illumination and fluorescein filters using procedures described in Masliah, *supra* (1991). The results were further confirmed by immunoelectron microscope analysis as described in Masliah, *supra*, (1991). Briefly, vibratome sections were blocked with normal goat serum (5%) and incubated overnight at 4°C with antibodies anti-X1 and anti-Y. The sections were washed in PBS, incubated with biotinylated goat anti-rabbit IgG followed by avidin D-HRP (Vector ABC Elite, Vector Labs, Inc., Burlingame, CA) and reacted with diaminobenzidine (DAB, 0.2 mg/ml) in 50 mM Tris buffer (pH 7.4) with 0.001% H_2O_2. Control sections were incubated with preimmune serum. The immunostained sections were postfixed for 20 minutes in 1% OsO_4, dehydrated, and embedded to present a flat surface in epoxy/Araldite. Ultrathin sections were cut with a Reichert OM-U3 ultramicrotome and viewed with a 100 CX JEOL electron microscope.

Results of the immunohistochemical and immunoelectron microscope analysis of AD brain sections are shown in FIGURE 1. Panel A shows hippocampal sections stained with antibodies anti-X1 and anti-Y. In Panel B occasional staining of dystrophic neutites (arrows) was detected with anti-Y antibody. Immunostaining of amyloid in diffuse, primitive, and mature plaques was detected in Panels A and B. as well as in cerebral vessel walls (not shown) as revealed by double staining with thioflavin S. When these sera were preabsorbed with fragments X1 and Y, staining was eliminated. As shown in Panel C, neither preimmune sera nor antisera containing anti-X1 and/or anti-Y antibodies stained amyloid in AD brain tissue when antisera had been preabsorbed with the corresponding peptide fragments X1 and Y. Panel D shows an electron micrograph of staining of amyloid fibrils (arrows) with anti-X1 antibody. Amyloid fibrils were also stained with the anti-Y antibody (not shown).

The anti-Y antibody stained not only amyloid in plaques, but also nuclei of small, possibly glial cells, cytoplasm of some small cells, and neuropil threads. The staining of these structures other than amyloid was not observed with the anti-X1 antibody. There are two potential explanations for the difference in staining properties between the anti-X1 and -Y peptide antibodies. This difference in staining may represent a genuine difference in the distribution of two products of a single precursor protein or, alternatively, it may be a result of a less specific staining by the anti-Y peptide antibody. It appears likely that the latter explanation is correct because anti-Y antibody stains dozens of bands on Western blots of brain extract (data not shown).

Recent work has shown that 50% of intracellular NFTs and 100% of extracellular MFTs contain Aβ. (G. Perry, *et al., Am. J. Pathol.*, 140:283-290, 1992). However, the thioflavin-positive NFTs did not stain positively with anti-X1 or -Y antibodies.

Immunoelectron microscopic analysis of QsO_4 intensified diaminobenzidine staining by anti-X1 antibody showed specific localization on amyloid fibrils (Fig. 1D). Anti-Y antibody also stained amyloid fibrils (not shown). These results indicate that both X and Y peptides are tightly associated with the amyloid fibrils. The data are compatible with the hypothesis that NAC is actually a novel amyloid component and not a component of contaminants such as paired helical filaments.

EXAMPLE 3

MOLECULAR CLONING OF NACP CDNA

To discover whether the NAC peptides are produced from a precursor protein as is the case with Aβ, the decision was made to isolate the cDNA. First a piece of cDNA for encoding the NAC peptide was deduced and amplified by PCR as described in R. K. Saiki, *et al.* (*Science*, 230:1350-1354, 1985). Sense (X1) (SEQ. I.D. NO. 9) and antisense (X2) (SEQ. I.D. NO. 10) oligonucleotides were designed as primers for the N- and C-terminal halves of the X peptide amino-acid sequence, respectively.

An additional set of sense Z (SEQ. I.D. NO. 11) and antisense αZ (SEQ. I.D. NO. 12) oligonucleotide primers for DNA flanking the EcoRI cloning site of λgt11 were made. PCR was performed using these oligonucleotides as primers with combinations of X1 (or X2) and Z (or αZ) as primers and a cDNA expression library of human brain tissue in λgt11 as template. Briefly, the PCR reaction was performed at 94°C for 1 minute, at 51°C for 1.5 minute, and at 72°C for 2 minutes. After 35 cycles of amplification, a PCR DNA product of about 280 bp obtained from the combination of primers X2 and αZ was purified, digested with EcoRI, subcloned into the EcoRI-HincII site of pBluescript SK+ (Stratagene, San Diego, CA), and sequenced using methods well known in the art.

An amino acid sequence deduced from the DNA sequence thus obtained was found to contain a ten amino acid sequence (EQATNAGGVA) constituting the N-terminal region of X peptide, thus confirming the identity of this PCR product as a fragment of NACP cDNA. With the origin of the PCR product confirmed, the DNA fragment was used as a probe for screening a cDNA expression library of human brain tissue (ATCC, #37432) in λgt11 phage.

From 5 x 10^5 recombinants, 22 positive clones were obtained. The isolated cDNA were subcloned into pBluescript SK+ and sequenced on both strands by Sanger dideoxy chain termination method (Sanger et al. 1977) using Sequenase (U. S. Biochemical Co., Cleveland, OH) with the help of synthetic primers. Clone HBS6-1 contained an apparently full-length cDNA with a nested sequence corresponding to NAC sequence.

Bacterial Expression of NACP Protein.

NACP protein was expressed in *E. coli* using pSENACP expression vector. Plasmid pHBS6-1 was digested with *Afl*II, treated with Klenow polymerase to generate a blunt end, and then digested with *Noc*I to release the coding region. This 1.2-kb *Nco*I-*Afl*II NACP cDNA fragment containing the entire coding sequence and 3' nontranslated region was ligated into a bacterial expression vector, pSE380 (Invitrogen), previously linearized by digestion with *Nco*I and *Sma*I. Resultant pSENACP expresses NACP protein under the control of trp/lac fusion promoter which is inducible with IPTG. pSENACP was amplified in *E. coli* HB101.

Northern Blot Analysis.

Procedures for RNA preparation, electrophoresis, and hybridization are those routinely used. Briefly, total RNA was isolated from different human tissues. RMA (10 μg) was electrophoresed on a 1% formaldehyde-agarose gel and blotted to a nitrocellulose membrane. Hybridization was carried out in 50% formamide, 5 x SSPE, 5 x Denhardt's, 0.5% SDS, 100μg/ml denatured salmon sperm DNA, and 10% dextran sulfate at 42°C, and exposed for 3 days at

-80°C. A higher stringency wash of 0.1 x SSC, 0.1% SDS at 65°C was also employed.

Sequence analysis of the HBS6-1 clone revealed a 420 bp open-reading frame DNA sequence encoding 140 amino-acid residues with a calculated relative molecular mass (M_r) of 14,459 (SEQ. I.D. NO. 1). As shown in FIGURE 2A, the sequence surrounding the predicted initiator methionine codon (GCCATGG) agrees with the Kozak consensus sequence as described by K. Kozak (*Nucleic Acid Res.*, 15:8125-8148, 1987). Also as shown in FIGURE 2A, the nearest in-frame stop codon was found 18 bp upstream of this ATG. The nearest in-frame stop codon (TAA) upstream to the putative initiation methionine codon is marked by an asterisk. The termination codon is marked by two asterisks. Sequences for X and Y peptides are boxed. Polyadenylation signals are underlined.

The deduced amino acid sequence shows that the X and Y peptides are located immediately next to each other in the middle of the precursor protein NACP. Neither an apparent signal peptide sequence nor canonical N-linked glycosylation sites were found.

EXAMPLE 4

1. STUDIES OF THE AMINO ACID SEQUENCE TO DETERMINE INDICATIONS PREDICTIVE OF SECONDARY STRUCTURE.

Using the methods of P. Y. Chou, *et al., Annu. Rev. Biochem.*, 47:251-276 (1978); G. D. Rose, *Nature*, 272:586-590 (1978); J. Garnier, *et al., J. Mol. Biol.*, 120:97-120 (1978), studies were conducted to determine sequence features predictive of secondary structure. These studies indicate that the NAC peptide sequence has a strong tendency to form a β-sheet configuration, as does Aβ.

2. HYDROPATHY TESTING OF THE PRECURSOR PROTEIN NACP.

Using the method of Kyte and Doolittle (J. Kyte and R.F. Doolittle, *J. Mol. Biol.*, 157:105-132, 1982) an analysis of NACP was performed using a window size 9 and Prosis software from Pharmacia (Piscataway, NJ). The main hydrophobic domain of the deduced amino acid sequence (amino acids 62 through 90) was located within the sequence of the NAC proteins X and Y (amino acids 61 through 95). When the NAC protein was synthesized, aggregates and precipitates formed easily in aqueous solutions. These results indicate that the precursor protein NACP is considerably more soluble in aqueous solutions (cytoplasm and other bodily fluids) than are the X and Y peptides associated with amyloid formations.

EXAMPLE 5

WESTERN BLOT ANALYSIS OF NACP PROTEIN.

Tissue homogenates were prepared from cytosolic fractions of frontal cortex from patients with typical and neuropathological features of AD obtained from the Alzheimer's Disease Research Center (San Diego, CA). Procedures for human brain preparation, electrophoresis, and immunoblotting detection have been reported by E. Masliah, *et al.* (*J. Neurosci*, 10:2113-2124, 1990). Briefly, proteins were electrophoresed on a 16% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting detection was performed using anti-X1 antibody (1:1500 dilution) and ^{125}I-protein A. To absorb anti-X1 antibody, 200 μg/ml X1 fragment (SEQ. I.D. NO. 6) or the control peptide (EGYQDYEPEAC) (SEQ. I.D. NO. 8) were used.

Western blot analysis with anti-X1 antibody detected NACP as a M_r 19K protein mostly in the cytosolic fractions as shown in FIGURE 3, lane 4. Lanes 1 and 6 of FIGURE 3 show *E. coli* transfected with pSE380 vector as control; lanes 2 and 5 show *E. coli* transfected with pSENACP expressing NACP; and lanes 3 and 4 show normal human brain. A shorter exposure time was employed for lanes 1 to 3 compared with lanes 4 to 6 because of high background. However, the M_r 19K band was not observed in lanes 2 and 3 even after a 4 times longer exposure.

The anti-Y antibody stained dozens of bands in addition to the M_r 19K band (data not shown). Protein staining of the M_r 19K band was abolished when either the anti-X1 or the anti-Y antibody was preabsorbed with its corresponding peptide fragment as shown in FIGURE 3, lane 3. However, the anti-X1 positive M_r 40 band was not blocked by preabsorption and was not detected with anti-Y antibody, indicating that this band is not specific to NACP.

To confirm the identity of the protein derived from brain homogenates, a NACP protein derived from the cDNA isolated in Example 3 above was produced *in vitro*. pHBS6-1 was digested with AflII, treated with Klenow polymerase to generate a blunt end, and then digested with NcoI to release the coding region. This 1.2kb NcoI-AflII NACP cDNA fragment of Sequence I. D. No. 1 containing the entire coding sequence and 3'-nontranslated region was ligated into a bacterial expression vector, pSE380 (Invitrogen, San Diego, CA), previously linearized by digestion with NcoI and SmaI. The resultant vector pSENACP expresses NACP protein under the control of trp/lac fusion promoter which is inducible with IPTG. pSENACP was amplified in E. coli HB101 using techniques as described in Sambrook, *supra* and others. A vector pSE380 without NACP cDNA was also transfected in E coli as a control. In Western Blot analysis as shown in FIGURE 3, lane 3, the bacterially expressed protein of M_r 19K comigrated with the homogenate-derived protein, confirming the identity of the homogenate-derived protein.

EXAMPLE 6

SEQUENCE ANALYSIS OF NACP PROTEIN

The amino acid sequence of NACP was examined for distinctive structural features. As shown in FIGURE 4A, the NACP protein is characterized by repetitive motifs. The KTKEGV motif is repeated seven times, but the amino acid positions 2 to 6 are sometimes substituted. In addition, as shown in FIGURE 4B, amino acids 48-56 and 70-78 of the NACP protein are homologous.

In addition to these repetitive motifs within the NACP protein, a computer homology search (FASTA program, in the UCSD BAX/VMS DNA protein sequence analysis system) of the DNA sequence data base (EMBL/GenBank Libraries) has discovered homology between NACP and EST01420, a protein recently identified by random sequencing of human brain cDNA (M. D. Adams, *et al., Nature*, 355:632-634, 1992). Comparison of the DNA encoding these proteins expressed in the human brain, as shown in FIGURE 4C, showed 74% identity in 124 nucleotides resulting in 80% identity in a 41 amino acid residue from the initiation methionine. Therefore, the two cDNAs are substantially homologous in the N-terminal region, but the EST01420 sequence has the termination codon at base pair position 206, and, therefore, could encode only 51 amino acids. The high degree of homology in the cDNA encoding these peptides expressed in the brain suggests that NACP may be a member of a heretofore unknown gene family.

EXAMPLE 7

NORTHERN BLOT ANALYSIS OF NACP MRNA

Procedures for RNA preparation, electrophoresis, and hybridization have been described previously (T. Saitoh, *et al.*, *Cell*, 58:615-622, 1989). Briefly, total RNA was isolated from different human tissues as described in Chirgwin, J.M., *et al.* (*Biochemistry*, 18:5294-5299, 1979). RNA (10 μg) was electrophoresed on a 1% formaldehyde-agarose gel and blotted to a nitrocellulose membrane. Hybridization was carried out in 50% formamide, 5 x SSPE, 5 x Denhardt's, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate at 42°C for 15 hr with ^{32}P-labeled 1.1-kb NACP cDNA which includes the X and Y sequence with a 3'-noncoding sequence. The membrane was washed with 0.1 x SSPE, 0.1% SDS at 42°C, and exposed for 3 days at -80°C. A higher stringency wash of 0.1 x SSC, 0.1% SDS at 65°C was also employed. A Northern blot was obtained from Clontech (Palo Alto, CA, #7760-1, Lot 32409). Hybridization and washing conditions for this blot followed the suggestions of the manufacturer.

As shown in FIGURE 5, Panel A was probed with NACP cDNA and, as a control, panel B was probed with glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA (ATCC, #57090). Briefly, the membrane was washed with 0.1 x SSPE, 0.1% SDS at 42°C, and exposed for 3 days at -80°C. The same pattern of signals was obtained after a higher stringency wash with 0.1 x SSC, 0.1% SDS at 65°C.

Two principal transcripts of 3.6 and 1.5 kb were found most enriched in brain, with lower concentrations in all tissues examined except in liver as shown in FIGUREs 5A and 5B. Comparable patterns of mRNA were observed in both normal and AD neocortex and cerebellum as shown in FIGURE 5D. Since HBS6-1 is 1560 nucleotides long it probably corresponds to the shorter transcript. A minor 1 kb band visible in FIGURE 5 D is believed to represent a transcript with a shorter 3' non-coding region resulting from the use of polyadenylation signals at 1023 bp or 1079 bp. It was discovered that the ratio of the different-sized transcripts varies depending on the age and origins of tissue employed as shown in FIGURE 5. The presence of the 3.6 kb transcript for NACP may be explained by an alternative splicing mechanism that is under developmental and/or tissue specific regulation.

EXAMPLE 8

NAC PEPTIDE AGGREGATION AND WESTERN BLOT ANALYSIS

A solution of synthetic NAC peptide (SEQ.ID.No.3) at a concentration of 600 μM was made in 50 mM boric buffer at pH 9.2. The solution was centrifuged at 100,000 x g for 1 hr and the supernatant was collected, neutralized by 1.5 N hydrochloride in 10 x PBS, and diluted in 1 x PBS (pH 7.4).

The aggregation of synthetic NAC peptide was followed by measurement of turbidity according to techniques known in the art (see, e.g., Jarrett, *et al.*, *Biochemistry*, 31:12345-12352 (1992); and, Jarrett, *et al.*, *Cell*, 73:1055-1058 (1993)). Turbidity was measured at 400 nm daily for 7 days under the following two conditions: 1) various concentrations of NAC peptide: 10, 30, 100, 300 μM at 37°C, and 2) 300 μM of NAC peptide at various temperatures: 4°, 22°, and 37°C. The peptide solutions/suspensions were mixed gently before each absorbance measurement.

The size of the aggregated NAC peptide was estimated by Western blot using anti-NAC-X1. Six aliquots of peptide solution were prepared in boric buffer, lyophilized, and dissolved in water, neutralized, and diluted with PBS (phosphate buffered saline) to a final concentration of 300 μM. They were kept at 37°C for 0, 1, 2, 3, 5, or 7 days, dissolved in Laemmli sample buffer (Laemmli, U. K., *Nature*, 227:680-685 (1970)), electrophoresed on 16% Tricine-SDS-polyacrylamide gel, and transferred to PVDF membrane for Western blot analysis.

NAC peptide was solubilized in 50 mM boric buffer (pH 9.2) although it was also soluble in formic acid and 6 M guanidinium thiocyanate. NAC peptide aggregated in distilled water and PBS. Turbidity at 400 nm of NAC peptide solution in PBS increased with time. As shown in FIGURE 6 A, this increase was dependent on both the concentration of NAC peptide and the temperature (FIGURE 6 B). At Day 1, 300 μM NAC peptide was required for the appreciable aggregation, whereas at Day 2 100 μM NAC peptide started to aggregate. The aggregation of NAC peptide at less than 30 μM was never remarkable. The aggregation of NAC peptide at 22 °C was comparable to that at 37 °C, although at 4 °C the aggregation was markedly delayed.

In Western blot, the monomer NAC peptide migrated to the apparent molecular mass of 3500 Da (FIGURE 7). The signal intensity of the 3500 Da band was significantly decreased on Day 5 and 7. Aggregated NAC peptide was found at the top of the gel from Day 1. This signal increased to a maximum at Day 2-3. No intermediate-size bands detected by anti-NAC-X1 were observed. Thus, anti-NAC-1 recognized NAC peptide but not its precursor, NACP, in both dot and Western blot analysis. These findings indicate that this antiserum is sensitive to both the sequence and the conformation, allowing the detection of only NAC peptide but NACP that surely contains the NAC peptide sequence. However, anti-NAC-X1 is not simply the conformational antibody, because it did not detect Ab blotted under the comparable conditions. Anti-NAC-X1 stained amyloid cores in AD brain. These data indicate that NAC fragments, which are shorter than NACP and have the similar structure as NAC peptide, accumulate in amyloid, and that they are immunologically identical to NAC peptide but distinct from NACP.

EXAMPLE 9

CONGO RED STAINING AND BIREFRINGENCE

Four hundred microliters of NAC peptide solution/suspension (300 μM) at pH 7.4 was stored over 2 weeks at 37°C to promote its aggregation. The solution/suspension was centrifuged at 16,000 x g for 30 min. The precipitate of peptide fibrils was collected, mixed in 1 ml of PBS solution containing 1 mM Congo red for 1 min, centrifuged at 16,000 x g for 5 min, and recollected. It was rinsed with 1 ml of distilled water for 1 min, and centrifuged at 16,000 x g for 5 minutes. The peptide precipitate was placed on a glass microscope slide and allowed to dry. Birefringence was determined with an Olympus fluorescence microscope (model BHF) equipped with a polarizing filter apparatus (model BH-POL).

The NAC peptide aggregate was stained by Congo red. It exhibited green-gold birefringence when viewed with bright-field (FIGURE 8 A) and cross-polarization (FIGURE 8 B) microscopy. The stained peptides appear in the FIGURES as bright patches.

EXAMPLE 10

ELECTRONMICROSCOPIC OBSERVATION

Electron microscopy of aggregated NAC peptide revealed clusters of fibrils deposited from the peptide suspension (FIGURE 9). The diameter of fibrils was generally about an Å. The repeated structures were found in the fibrils. The structure of NAC peptide fibrils was different from that of Ab in AD brain.

Based on the above-referenced data, it was determined that the NAC peptide has the following characteristics of amyloid: 1) green birefringence after Congo red staining when viewed with a polarizing microscope; 2) a typical structure under the electron microscope (fine, rigid, nonbranching fibrils); 3) insolubility in aqueous solution. Further, NAC was determined to be self-aggregating.

However, it is important to note that the amyloid found in the brain tissue of patients with AD when viewed with electron microscopy is different from that formed by NAC aggregation. Thus, it is likely that NAC can not explain the global process of amyloid formation in AD. Rather, it is speculated that NAC is involved in only the initial process of amyloid formation and the major process of amyloidogenesis is due to the accumulation of Ab aggregation. Thus, NAC may serve as a seed to form amyloid as a minor component, on which Ab aggregate as a major component of amyloid. Further investigation will investigate the possibility that NAC may contribute to the process of Ab aggregation and amyloid forming and maturing.

EXAMPLE 11

DISTRIBUTION OF NACP IN RAT BRAIN SECTIONS

Using the streptavidin-biotin-peroxidase (SAB) staining method, rat brain sections were stained by anti-NACP(131-140; SEQ.ID.No.6) and anti-NACP(1-9; SEQ.ID.No.8) antisera. Briefly, Sprague-Dawley rats weighing 250 - 300 g were deeply anesthetized by sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL), perfused transcardially with 4% paraformaldehyde in PBS for 20 min, and the brain was removed and immediately placed in a postfixative, 4% paraformaldehyde solution, for 4 days at 4°C. The brain was immersed in a 30% sucrose solution in PBS for 4 days at 4°C, frozen in -40°C hexane, and cut into 20-μm sections using a cryostat at -20°C.

Immunohistochemistry was performed according to the modified SAB method. Sections were rinsed for 3 x 5 min in PBS, incubated for 10 min in PBS including 0.1% Triton X-100, and incubated for 20 min with 3% H2O2 to inhibit endogenous peroxidase. They were then incubated for 10 min with 10% normal goat serum (NGS) (Nichirei, Tokyo, Japan) in PBS and incubated for 18 hr at 4°C with anti-NACP(131-140) or anti-NACP(1-9) antiserum in PBS containing 1% BSA. Sections were rinsed for 3 x 5 min in PBS and incubated with biotinylated goat anti-rabbit-IgG solution (Nichirei, Tokyo, Japan) for 10 min. They were then rinsed for 3 x 5 min in PBS, incubated with streptavidin-peroxidase solution (Nichirei, Tokyo, Japan) for 5 min, and rinsed for 3 x 5 min in PBS. NACP-positive structures were visualized by incubating the tissue in 0.05% diaminobenzidine with 0.01% H_2O_2 in 0.61 M Tris/HCl buffer (pH 7.4) for 5 - 15 min. Specificity of the immunohistochemical reaction was confirmed by the absence of staining in adjacent tissue sections incubated with preabsorbed antiserum.

Double-immunostaining was performed with antisera against NACP and synaptophysin as described previously (Masliah, *et al., Exp.Neurol.*, 113:131-142, 1991). After treatment with 0.1% Triton X-100 and 3% H_2O_2, sections were incubated for 1 hr with 5% normal horse serum (NHS) (Vector Labs, Inc., Burlingame, CA), 10% NGS (Vector Labs, Inc.), and 2% BSA in PBS. They were then incubated for 18 hr at 4°C with the mixture of mouse monoclonal antibody against synaptophysin (Sy38, Boehringer Mannheim, Indianapolis, IN) (Wiedenmann and Franke, *Cell*, 41:1017-1028, 1985) and rabbit polyclonal antisera, anti-NACP(131-140) or anti-NACP(1-9), in PBS containing 3% NHS, NGS, and BSA. Sections were rinsed for 3 x 5 min in PBS, incubated for 1 hour with biotinylated goat anti-rabbit-IgG in PBS including 1% BSA, and rinsed for 3 x 5 min in PBS. They were then incubated for 80 minutes with Texas-red-labeled avidin (Vector Labs, Inc.) and fluorescein isothiocyanate (FITC)-labeled horse anti-mouse-IgG antibody (Vector Labs, Inc.) in PBS including 1% BSA and rinsed for 3 x 5 min in PBS. The double-labeled sections were covered with glass coverslips with antifading medium (Vector Labs, Inc.).

These sections were observed with the Bio-Rad MRC-600 laser confocal scanning microscope mounted on a Nikon Optiphot microscope. This system permits the simultaneous analysis of double-labeled samples in the same optical plane. The digitized video images of serial 1-μm optical sections were stored on an optical disk for subsequent processing and analysis.

For electron microscopic study, 40-μm vibratome sections were blocked with NGS (5%) and incubated overnight at 4°C with anti-NACP(131-140). The sections were washed in PBS, incubated with biotinylated goat anti-rabbit IgG followed by avidin D-HRP (Vector ABC elite, Vector Labs, Inc.), and reacted with diaminobenzidine (DAB; 0.2 mg/ml) in 50 mM Tris buffer (pH 7.4) with 0.001% H2O2. The immunostained sections were postfixed for 20 min in 1% OsO4, dehydrated, and flat embedded in epoxy/Araldite. Ultrathin sections were cut with a Reichert OM-U3 ultramicrotome and viewed with a 100 CX JEOL electron microscope.

The intensity of staining with anti-NACP(131-140) was relatively strong in the gray matter of the cerebral cortex (layers II, III, and V), anterior olfactory nucleus, caudate putamen, nucleus accumbens, hippocampus, ventral tegmental area, substantia nigra, pontine nuclei, and cerebellar cortex as shown macroscopically in FIGURE 10 A. The staining pattern of sections using anti-NACP(1-9) antisera was identical to that of anti-NACP(131-140). This staining was abolished by preincubation of the antisera with the corresponding peptide.

The results of microscopic observation of the sections stained using the SAB method are shown in FIGURES 10 B-D. In cerebellum (FIGURE 10 B), the molecular layer and granule cell layer were stained, but the medullary layer and the Purkinje cell perikarya were not stained. In both the molecular and granule cell layers, the perikarya of cells, including stellate, basket, Golgi, granule, and glial cells were not stained. An intermediate region where neurites were extended was stained with a "punctate" pattern. In hippocampus (FIGURE 10 C) and cerebral cortex (FIGURE 10 D), cell perikarya were not stained and neurites were stained showing "punctate" staining pattern similar to the molecular layer of the cerebellum. No ependymal or mantle cells were stained in any region.

Laser scanning confocal microscopic analysis of sections double immunolabeled with antibodies against NACP and synaptophysin showed that both markers colocalized in the great majority of the presynaptic terminals (FIGURES 11 and 12). In the neocortex, hippocampus, basal ganglia, olfactory region, and thalamus, between 70 and 100% of the axosomatic, axoaxonic, and axodentritic terminals contained both markers (FIGURE 12). In contrast, in the deep cerebellar nuclei and brain stem nuclei, although synaptophysin and NACP colocalization in axodendritic terminals was relatively common, only a small proportion of the axosomatic terminals contained NACP (FIGURES 11 and 12). In both cortical and subcortical regions a small proportion of terminals was synaptophysin positive and NACP negative (FIGURE 12). Occasionally, punctate structures displayed NACP immunoreactivity but not synaptophysin reactivity. Serial section analysis showed that these neuritic structures actually corresponded to the terminal segment of axons. Abundant NACP immunoreactivity was observed associated with specialized synaptic complexes including the glomeruli of the olfactory bulb (FIGURE 11 D-F) and the glomeruli of the cerebellar granular layer (FIGURE 11 G-I). At the ultrastructural level (shown by immunoelectron microscopy), the membrane of synaptic vesicles in presynaptic terminals were stained by anti-NACP(131-140) (FIGURE 13).

EXAMPLE 12

QUANTIFICATION OF NACP, APP AND SYNAPTOPHYSIN IN RAT BRAIN SECTIONS

Brains of young adult Sprague-Dawley rats weighing 250 - 300 g were separated into 10 portions as follows: olfactory bulb, frontal cortex, striatum, hippocampus, hypothalamus, thalamus, midbrain, cerebellum, pons & medulla oblongata, and pituitary gland. For APP and synaptophysin quantification, 40 μg protein of either cytosolic or particulate fraction was loaded on a 10% SDS-polyacrylamide gel and blotted to nitrocellulose membrane. The mouse monoclonal antibody against the N-terminal of APP (22C11) (Boehringer Mannheim) (Weidemann, *et al.*, *Cell*, 57:115-126, 1989) or SY38 (Boehringer Mannheim) in PBS, including 1% BSA and 0.1% Tween20, was used as the primary antibody, followed by rabbit anti-mouse IgG polyclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) at the dilution of 1:2000 in PBS including 3% BSA for 1 hr, then incubated with 0.5 μCi/ml iodinated protein A and apposed to Kodak X-Omat RP film at -80°C.

Films were then developed with a Konica film developer and scanned with an LKB densitometer for quantification of the NACP bands and APP bands. X-ray film was exposed to membrane to give bands in the OD range between 0.8 and 2.5 where the sensitivity of the film is relatively linear.

Immunoreactive NACP protein bands were detected as a molecular mass of 19,000 Da in the cytosolic fraction of brain homogenate. Since APP protein bands were observed at molecular masses of 75,000 - 105,000 Da in the cytosolic fraction and at molecular masses of 100,000 - 115,000 Da in the particulate fraction by 22C11, a monoclonal antibody against APP (Weidemann, *et al.*, 1989, *supra*), the sum of signal intensity in both fractions was used as the amount of APP. Synaptophysin protein bands were observed with a molecular mass of 38,000 Da in the particulate fraction of brain sample by a mouse monoclonal antibody, SY38.

As shown in FIGURES 14 through 16, the concentration of NACP was high in the telencephalon (end-brain areas), including the olfactory bulb, frontal cortex, striatum, and hippocampus, intermediate in the hypothalamus and thalamus, and low in the midbrain, cerebellum, pons & medulla oblongata, and pituitary gland. APP and synaptophysin were more evenly distributed in most portions of brain. However, the amount in the pituitary gland was small.

EXAMPLE 13

ALTERATION OF NACP IN AD BRAIN TISSUE

As described in Example 1, AD brain tissue was obtained from the frontal cortex of patients with typical clinical and neuropathological features of AD (supplied by the Alzheimer's Disease Research Center, San Diego, CA). Using the double immunolabeling and laser confocal microscopy techniques described in the preceding Examples, the quantity of NACP in presynaptic terminals, as well as diffuse and mature amyloid plaques, was compared in tissue from AD brain and "normal" brain tissue.

Interestingly, the number of NACP containing presynptic terminals is significantly decreased in AD brain tissue as compared to normal brain tissue--by 30-40% (FIGURES 15 through 16). Further, although the overall synaptic populations are decreased in AD brain tissue, there is a significant increase in the quantity of NACP present in each presynaptic bouton of AD brain tissue, indicating a compensatory mechanism for the loss of synapses (FIGURES 15 through 16).

In addition, anti-NAC antibodies (as described elsewhere above) bound about 35% of the diffuse plaques and about 55% of the mature plaques present in the AD brain tissue tested (FIGURE 17). Double immunolabeling of tissue with anti-β-amyloid antibodies and anti-NAC antibodies showed that NAC is also more abundant than β-amyloid in AD brain tissue (FIGURE 18). Further, control tissue samples obtained from elderly persons without AD but whose brain tissue contained relatively small groups of diffuse plaques did not react with anti-NAC antibodies, while tissue samples obtained from persons suffering from either early or advanced cases of AD that contained a relatively large number of diffuse plaques as well as mature plaques reacted strongly with anti-NAC antibodies in about 30-50% of the plaques (FIGURES 16 and 18). These studies indicate that there is a connection between metabolism of presynaptic proteins (e.g., NACP) and plaque formation, and that NAC accumulation (in conjunction with β-amyloid accumulation) leads to the evolution of diffuse plaques into mature ones.

EXAMPLE 14

NACP HOMOLOGIES

As discussed in the Detailed Description of the Invention, a computer search of the DNA sequence data base (EMBL/ GenBank Libraries) revealed that some proteins had homologous sequence to NACP. Rat synuclein (SYN1) showed the highest homology; 7 amino acids were substituted in 140 amino acid sequence in NACP. More specifically, the NACP amino acid sequence showed 95% identity with that of rat synuclein 1, a synaptic/nuclear protein previously identified in rat brain, indicating that NACP is the human homologue of rat synuclein 1. Rat SYN2 also showed high homology to NACP, howevere, approximate 50 amino acid sequence of C-terminal was different. Rat SYN3 and human EST01420 identified by random sequencing of human brain cDNAs (Adams, *et al.*, *Nature*, 355: 632-634, 1992), had homologous sequence to N-terminal portion of NACP, but neither of them included NAC portion. *Torpede* synuclein also showed homologous sequence with 16 amino acids insertion between the residue 28 and 29 of NACP. Homo sapiens putatively transcribed partial sequence (HSPTPS), found by MRC Human Genome Mapping Project, had homologous sequence corresponding to NAC portion of NACP. These data suggest that the metabolic alterations of presynaptic proteins are associated with the amyloid and plaque formation in Alzheimer's disease.

EXAMPLE 15

ANIMAL MODEL FOR *IN VIVO* ADMINSTRATION OF β-AMYLOID PEPTIDES USEFUL IN TESTING THE ABILITY OF NAC/NACP PEPTIDES TO CROSS THE BLOOD/BRAIN BARRIER IN MAMMALS

β-amyloid peptides of differing lengths as described below were administered to rats to determine whether the peptides would cross the blood/brain barrier in mammals. The experimental animal models consisted of 6 groups as follows:

1) Rats injected unilaterally in the neocortex with 5 μl of full-length unlabeled β-amyloid (1-40, 0.01 mM, Bachem), followed 1 day later by intracarotid injection of ^{125}I substance P (100-500 pmol, NEN) or ^{125}I β-A peptide (1-28, 5 nmol, unlabeled peptide from Sigma Chemical Co. and iodination using DuPont's NEN iodination product); 2) rats injected unilaterally in the neocortex with 5 μl of unlabeled β-amyloid (1-40, 0.01 mM) followed 1 day later by intracarotid injection of ^{125}I alone; 3) rats injected unilaterally in the neocortex with 5 μl of unlabeled β-amyloid (1-40, 0.01 mM), followed 1 day later by intracarotoid injection of ^{125}I substance P (100-500 pmol) or ^{125}I β-A peptide (1-28, 5 nmol); 4) rats injected unilaterally in the neocortex with 5 μl of sterile saline, followed 1 day later by intracarotid injection of ^{125}I substance P (100-500 pmol)

or ^{125}I β-A peptide (1-28, 5 nmol); 5) rats injected unilaterally in the neocortex with 5 μl of sterile saline, followed 1 day later by intracarotid injection of sterile saline; 6) rats injected unilaterally in the neocortex with 5 μl of unlabelled β-amyloid (1-40), followed 1 day later with labelled and unlabelled substance P and β-A peptide (1-28) injected in the carotid artery.

Control data was generated from cryostat sections taken from the cortex of normal and AD cases which were incubated with radiolabelled and unlabelled β-amyloid and substance P. Use of intracerebral injections of amyloid into the rat brain as an animal model mimics the deposits of amyloid in the plaques of the patients with AD.

Although this animal model is not a model of AD *per se*, it is a model of amyloid deposits in the brain, which is one of the most important diagnostic hallmarks of AD. This animal model will, therefore, be useful in testing whether NAC/NACP peptides administered as described with respect to β-amyloid peptides will cross the blood/brain barrier.

EXAMPLE 16

β-A PEPTIDE (1-28) PASSAGE ACROSS THE BLOOD/BRAIN BARRIER TO BIND UNLABELLED β-AMYLOID

Five hundred μl of ^{125}I β-amyloid (1-28) (5μCi/ml) were administered into the carotid arteries of rats. Five minutes after the injection about 100 CPM were measured in brain samples. Maximum counts in the brain were recovered 50 minutes after injection because the number of CPM recovered from the brain reaches a plateau at this point in time.

Rats that received direct injection of ^{125}Iβ-amyloid (1-28) into the brain displayed the highest CPM's recovered from the brain. Rats that received ^{125}I β-amyloid (1-28) into the carotid artery and that previously received an injection of unlabelled β-amyloid (1-40) into the neocortex displayed a 100 fold higher count in the brain compared with rats that received an injection of saline alone in the brain followed by intracarotid administration of ^{125}I β-amyloid (1-28). Control experiments where ^{125}I β-amyloid (1-28) was substituted by vehicle alone showed only background CPM's, thus indicating that ^{125}I β-amyloid (1-28) injected into the circulation crossed the blood/brain barrier and bound the unlabelled amyloid injected in the brain.

As discussed in the Detailed Description of the Invention, these results indicate that NAC/NACP peptides of 28 amino acids in length or shorter will also cross the blood/brain barrier.

The CPM measurements reported were obtained by spectrophotometric analysis of fresh samples of brain tissue (taken from living, anaesthetized rats). For comparison, cryostat sections were also taken from rats in each model group for *in vitro* analysis using the AMBIS 4000 radioimaging acquisition and analysis system (i.e., computed tomography system) (Ambis, San Diego, CA).

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, the following claims are intended to be interpreted to embrace all such modifications.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

(ii) TITLE OF INVENTION: NOVEL COMPONENT OF AMYLOID IN ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Spensley Horn Jubas & Lubitz
(B) STREET: 1880 Century Park East - Suite 500
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 90067

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE: 29-AUG-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Howells, Stacy L.
(B) REGISTRATION NUMBER: 34,842
(C) REFERENCE/DOCKET NUMBER: FD-3520

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 455-5100
(B) TELEFAX: (619) 455-5110

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1560 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: cDNA for NACP

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..1560

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GCTCTCGGAG TGGCCATTCG ACGACAGTGT GGTGTAAAGG AATTCATTAG CCATGGATGT     60

ATTCATGAAA GGACTTTCAA AGGCCAAGGA GGGAGTTGTG GCTGCTGCTG AGAAAACCAA    120

ACAGGGTGTG GCAGAAGCAG CAGGAAAGAC AAAAGAGGGT GTTCTCTATG TAGGCTCCAA    180

AACCAAGGAG GGAGTGGTGC ATGGTGTGGC AACAGTGGCT GAGAAGACCA AAGAGCAAGT    240

GACAAATGTT GGAGGAGCAG TGGTGACGGG TGTGACAGCA GTAGCCCAGA AGACAGTGGA    300

GGGAGCAGGG AGCATTGCAG CAGCCACTGG CTTTGTCAAA AAGGACCAGT TGGGCAAGAA    360

TGAAGAAGGA GCCCCACAGG AAGGAATTCT GGAAGATATG CCTGTGGATC CTGACAATGA    420

GGCTTATGAA ATGCCTTCTG AGGAAGGGTA TCAAGACTAC GAACCTGAAG CCTAAGAAAT    480

ATCTTTGCTC CCAGTTTCTT GAGATCTGCT GACAGATGTT CCATCCTGTA CAAGTGCTCA    540

GTTCCAATGT GCCCAGTCAT GACATTTCTC AAAGTTTTTA CAGTGTATCT CGAAGTCTTC    600
```

CATCAGCAGT GATTGAAGTA TCTGTACCTG CCCCCACTCA GCATTTCGGT GCTTCCCTTT 660

CACTGAAGTG AATACATGGT AGCAGGGTCT TTGTGTGCTG TGGATTTTGT GGCTTCAATC 720

TACGATGTTA AAACAAATTA AAAACACCTA AGTGACTACC ACTTATTTCT AAATCCTCAC 780

TATTTTTTTG TTGCTGTTGT TCAGAAGTTG TTAGTGATTT GCTATCATAT ATTATAAGAT 840

TTTTAGGTGT CTTTTAATGA TACTGTCTAA GAATAATGAC GTATTGTGAA ATTTGTTAAT 900

ATATATAATA CTTAAAAATA TGTGAGCATG AAACTATGCA CCTATAAATA CTAAATATGA 960

AATTTTACCA TTTTGCGATG TGTTTTATTC ACTTGTGTTT GTATATAAAT GGTGAGAATT 1020

AAAATAAAAC GTTATCTCAT TGCAAAAATA TTTTATTTTT ATCCCATCTC ACTTTAATAA 1080

TAAAAATCAT GCTTATAAGC AACATGAATT AAGAACTGAC ACAAAGGACA AAAATATAAA 1140

GTTATTAATA GCCATTTGAA GAAGGAGGAA TTTTAGAAGA GGTAGAGAAA ATGGAACATT 1200

AACCCTACAC TCGGAATTCC CTGAAGCAAC ACTGCCAGAA GTGTGTTTTG GTATGCACTG 1260

GTTCCTTAAG TGGCTGTGAT TAATTATTGA AAGTGGGGTG TTGAAGACCC CAACTACTAT 1320

TGTAGAGTGG TCTATTTCTC CCTTCAATCC TGTCAATGTT TGCTTTATGT ATTTTGGGGA 1380

ACTGTTGTTT GATGTGTATG TGTTTATAAT TGTTATACAT TTTTAATTGA GCCTTTTATT 1440

AACATATATT GTTATTTTTG TCTCGAAATA ATTTTTTAGT TAAAATCTAT TTTGTCTGAT 1500

ATTGGTGTGA ATGCTGTACC TTTCTGACAA TAAATAATAT TCGACCATGA AAAAAAAAAA 1560

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: NACP

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..140

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asp Val Phe Met Lys Gly Leu Ser Lys Ala Lys Glu Gly Val Val
1               5                   10                  15

Ala Ala Ala Glu Lys Thr Lys Gln Gly Val Ala Glu Ala Ala Gly Lys
            20                  25                  30

Thr Lys Glu Gly Val Leu Tyr Val Gly Ser Lys Thr Lys Glu Gly Val
        35                  40                  45

Val His Gly Val Ala Thr Val Ala Glu Lys Thr Lys Glu Gln Val Thr
    50                  55                  60

Asn Val Gly Gly Ala Val Val Thr Gly Val Thr Ala Val Ala Gln Lys
65                  70                  75                  80

Thr Val Glu Gly Ala Gly Ser Ile Ala Ala Ala Thr Gly Phe Val Lys
                85                  90                  95

Lys Asp Gln Leu Gly Lys Asn Glu Glu Gly Ala Pro Gln Glu Gly Ile
            100                 105                 110

Leu Glu Asp Met Pro Val Asp Pro Asp Asn Glu Ala Tyr Glu Met Pro
        115                 120                 125

Ser Glu Glu Gly Tyr Gln Asp Tyr Glu Pro Glu Ala
    130                 135                 140
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: NAC

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Glu Gln Val Thr Asn Val Gly Gly Ala Val Val Thr Gly Val Thr Ala
1               5                   10                  15

Val Ala Gln Lys Thr Val Glu Gly Ala Gly Ser Ile Ala Ala Ala Thr
            20                  25                  30

Gly Phe Val
        35
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Peptide X

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Glu Gln Val Thr Asn Val Gly Gly Ala Val Val Thr Gly Val Thr Ala
1               5                   10                  15

Val Ala Gln Lys
            20
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Peptide Y

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Thr Val Glu Gly Ala Gly Ser Ile Ala Ala Ala Thr Gly Phe Val
1               5                   10                  15
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Fragment X1

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Glu Gln Val Thr Asn Gly Gly Ala Cys
1               5
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Fragment Y

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Thr Val Glu Gly Ala Gly Ser Cys
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Control Peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..11

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Gly Tyr Gln Asp Tyr Glu Pro Glu Ala Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Sense Primer (X1)

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 1..29
(D) OTHER INFORMATION: /note= "where N is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GARCARGTNA CNAAYGTNGG NGGNGCNGT 29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: antisense primer (X2)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..93

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 1..29
(D) OTHER INFORMATION: /note= "where N is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTYTGNGCNA CNGCNGTNAC NCCNGTNAC 29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Sense Primer (z)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..23

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACGACTCCTG GAGCCCGTCA GTA 23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Antisense Primer (alpha z)

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..23

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAATGGTAG CGACCGGCGC TCA 23

SUMMARY OF THE SEQUENCES

Sequence I.D. No. 1 oligonucleotide is a sequence for cDNA encoding NACP polynucleotide.

Sequence I.D. No. 2 is an amino acid sequence for NACP polypeptide.

Sequence I.D. No. 3 is an amino acid sequence for NAC polypeptide.

Sequence I.D. No. 4 is an amino acid sequence for X peptide.

Sequence I.D. No. 5 is an amino acid sequence for Y peptide

Sequence I.D. No. 6 is an amino acid sequence for fragment X1

Sequence I.D. No. 7 is an amino acid sequence for fragment Y

Sequence I.D. No. 8 is an amino acid sequence for a control peptide.

Sequence I.D. No. 9 oligonucleotide is a sense primer (X1) for the N-terminal half of X peptide.

Sequence I.D. No. 10 oligonucleotide is an antisense primer (X2) for the C-terminal half of X peptide.

Sequence I.D. No. 11 oligonucleotide is a sense primer (Z) for a region flanking the EcoRI cloning site of lambda gt11.

Sequence I.D. No. 12 oligonucleotide is an antisense primer (αZ) for a region flanking the EcoRI cloning site of lambda gt11.

CLAIMS

1. An isolated polypeptide comprising all or a portion of NACP.

2. An isolated polynucleotide which encodes the polypeptide of claim 1.

3. The polynucleotide of claim 2, wherein the polynucleotide is DNA.

4. The polynucleotide of claim 2, wherein the polynucleotide is RNA.

5. A host cell containing the polynucleotide of claim 2.

6. A recombinant expression vector containing the polynucleotide of claim 2.

7. The vector of claim 6, wherein the polynucleotide is an antisense sequence.

8. The vector of claim 6, which is a virus.

9. The vector of claim 8, wherein the virus is an RNA virus.

10. The vector of claim 9, wherein the RNA virus is a retrovirus.

11. The vector of claim 6, wherein the vector is a colloidal dispersion system.

12. The vector of claim 11, wherein the colloidal dispersion system is a liposome.

13. The vector of claim 12, wherein the liposome is essentially target specific.

14. The vector of claim 13, wherein the liposome is anatomically targeted.

15. The vector of claim 13, wherein the liposome is mechanistically targeted.

16. The vector of claim 15, wherein the mechanistic targeting is passive.

17. The vector of claim 15, wherein the mechanistic targeting is active.

18. The vector of claim 17, wherein the liposome is actively targeted by coupling with a moiety selected from the group consisting of a sugar, a glycolipid and a protein.

19. The vector of claim 18, wherein the protein moiety is an antibody.

20. The vector of claim 6, wherein the vector is a plasmid.

21. Antibodies which are immunoreactive with the polypeptide of claim 1, or fragments thereof.

22. The antibodies of claim 21, wherein the antibodies are polyclonal.

C 23. The antibodies of claim 21, wherein the antibodies are monoclonal.

24. A method for detecting a cell expressing NACP comprising contacting a cell component with a reagent which binds to the component.

25. The method of claim 24, wherein the component is nucleic acid.

26. The method of claim 24, wherein the component is protein.

27. The method of claim 25, wherein the nucleic acid is DNA.

28. The method of claim 25, wherein the nucleic acid is RNA.

29. The method of claim 24, wherein the reagent is a probe.

30. The method of claim 29, wherein the probe is nucleic acid.

31. The method of claim 29, wherein the probe is an antibody.

32. The method of claim 31, wherein the antibody is polyclonal.

33. The method of claim 31, wherein the antibody is monoclonal.

34. The method of claim 26, wherein the protein is NACP and the reagent is an NACP peptide.

35. The method of claim 34, wherein the NACP peptide is about 28 amino acids in length or shorter.

36. The method of claim 26, wherein the protein is NACP and the reagent is a NAC peptide.

37. The method of claim 36, wherein the NAC peptide is about 28 amino acids in length or shorter.

38. The method of claim 24, wherein the reagent is detectably labeled.

39. The method of claim 38, wherein the label is selected from the group consisting of a radioisotope, a paramagnetic isotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

40. A method for detecting amyloid formation in brain comprising contacting a brain tissue sample with a reagent which binds to NAC.

41. The method of claim 40, wherein the reagent is a probe.

42. The method of claim 41, wherein the probe is an antibody.

43. The method of claim 42, wherein the antibody is polyclonal.

44. The method of claim 42, wherein the antibody is monoclonal.

45. The method of claim 40, wherein the reagant is a NAC peptide.

46. The method of claim 45, wherein the NAC peptide is about 28 amino acids in length or shorter.

47. The method of claim 40, wherein the reagant is detectably labeled.

48. The method of claim 47, wherein the label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

49. A method of treating an amyloid disorder associated with NAC comprising administering to a subject with the disorder, a therapeutically effective amount of reagent which modulates NACP activity.

50. The method of claim 49, wherein the reagent is an antisense polynucleotide sequence.

51. The method of claim 50, wherein the reagent is an antibody.

52. The method of claim 51, wherein the antibody is monoclonal.

53. The method of claim 49, wherein the reagant is a NAC peptide.

54. The method of claim 53, wherein the NAC peptide is about 28 amino acids in length or shorter.

55. The method of claim 49, wherein the reagant is detectably labeled.

56. The method of claim 49, wherein the amyloid disorder is formation of neuritic plaques in the brain.

57. The method of claim 49, wherein the reagent is a sense polynucleotide sequence.

58. A method of treating an amyloid disorder associated with NAC comprising administering to a subject with the disorder, a therapeutically effective amount of reagent which modulates NAC aggregation.

59. The method of claim 58, wherein the reagant is a NAC peptide.

60. The method of claim 59, wherein the NAC peptide is about 28 amino acids in length or shorter.

61. The method of claim 59, wherein the reagant is detectably labeled.

62. The method of claim 58, wherein the amyloid disorder is formation of neuritic plaques in the brain.

63. An isolated polynucleotide sequence which comprises 5' and 3' untranslated nucleotide sequences associated with the nucleotide sequence which encodes NACP.

64. The polynucleotide of claim 63, wherein the polynucleotide is Sequence I.D. No 1.

65. An isolated polynucleotide sequence which comprises nucleotide sequence encoding NAC polypeptide, or a fragment thereof.

66. An isolated polypeptide wherein the polypeptide is encoded by the polynucleotide of Sequence ID. No. 1.

67. The polypeptide of claim 66, wherein the polypeptide is Sequence I.D. No. 3.

68. The polypeptide of claim 66, wherein the polypeptide is Sequence I.D. No. 4.

69. The polypeptide of claim 66, wherein the polypeptide is Sequence I.D. No. 5.

70. A transgenic mouse comprising a gene that encodes the polypeptide NACP, a fragment thereof, or a functional derivative thereof.

71. A method for diagnosing AD by detecting the presence of NAC in amyloid in the brain tissue of a mammal, comprising:

(a) administering a detectably labeled NAC peptide that will cross the blood/brain barrier of the mammal and bind any NAC therein into the blood circulation of the mammal; and,

(b) detecting any binding of NAC by the detectably labeled NAC peptide.

72. The method according to claim 71 wherein the NAC peptide is about 28 amino acids in length or shorter.

73. The method according to claim 71 wherein the NAC peptide is detectably labeled with a radioisotope.

74. The method according to claim 73 wherein any bound, detectably labeled peptide is detected by single photon emission computed tomography or postiron emission tomography.

75. The method according to claim 71 wherein the NAC peptide is detectably labeled with a paramagnetic isotope.

76. The method according to claim 75 wherein any bound, detectably labeled NAC peptide is detected by magnetic resonance imaging.

77. A method for evaluating the progression of amyloid formation in AD by detecting the presence of NAC in amyloid in the brain tissue of a mammal, comprising:

(a) administering a detectably labeled reagent that will cross the blood/brain barrier of the mammal and bind any NAC therein into the blood circulation of the mammal; and,

(b) detecting any binding of NAC by the detectably labeled reagent.

78. The method according to claim 77 wherein the reagent is a NAC peptide.

79. The method according to claim 78 wherein the NAC peptide is about 28 amino acids in length or shorter.

80. The method according to claim 77 wherein the reagent is an antibody.

81. The method according to claim 77 wherein the reagent is detectably labeled with a radioisotope.

82. The method according to claim 81 wherein any bound, detectably labeled reagent is detected by single photon emission computed tomography or postiron emission tomography.

83. The method according to claim 77 wherein the reagent is detectably labeled with a paramagnetic isotope.

84. The method according to claim 83 wherein any bound, detectably labeled reagent is detected by magnetic resonance imaging.

85. A method for evaluating the progression of amyloid formation in AD by detecting the presence of NACP in amyloid in the brain tissue of a mammal, comprising:
   (a) administering a detectably labeled reagent that will cross the blood/brain barrier of the mammal and bind any NACP therein into the blood circulation of the mammal; and,
   (b) detecting any binding of NACP by the detectably labeled reagent.

86. The method according to claim 85 wherein the reagent is a NACP peptide.

87. The method according to claim 86 wherein the NACP peptide is about 28 amino acids in length or shorter.

88. The method according to claim 85 wherein the reagent is an antibody.

89. The method according to claim 85 wherein the reagent is detectably labeled with a radioisotope.

90. The method according to claim 89 wherein any bound, detectably labeled reagent is detected by single photon emission computed tomography or postiron emission tomography.

91. The method according to claim 85 wherein the reagent is detectably labeled with a paramagnetic isotope.

92. The method according to claim 91 wherein any bound, detectably labeled reagent is detected by magnetic resonance imaging.

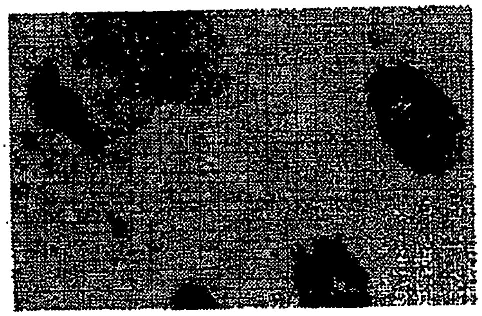


Fig. 1A

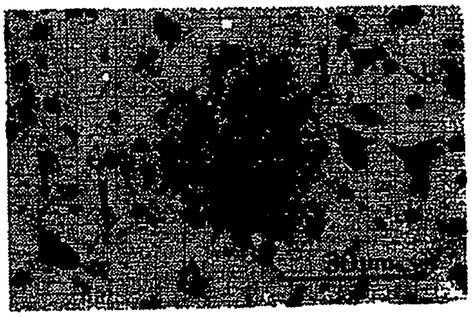


Fig. 1B

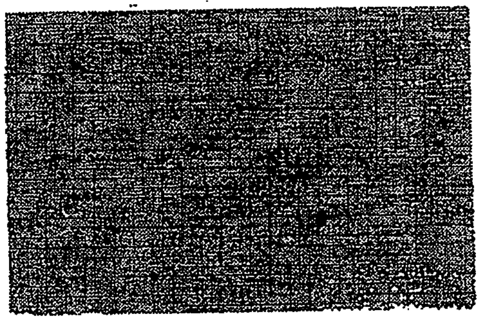


Fig. 1C

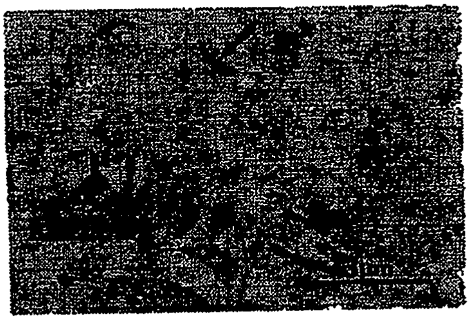


Fig. 1D

```
  1     G CTC TCG GAG TGG CCA TTC GAC GAC AGT GTG GTG TAA AGG AAT TCA    46
                                                   *
 47   TTA GCC ATG GAT GTA TTC ATG AAA GGA CTT TCA AAG GCC AAG GAG GGA    94
  1            M   D   V   F   M   K   G   L   S   K   A   K   E   G    14

 95   GTT GTG GCT GCT GCT GAG AAA ACC AAA CAG GGT GTG GCA GAA GCA GCA   142
 15    V   V   A   A   A   E   K   T   K   Q   G   V   A   E   A   A    30

143   GGA AAG ACA AAA GAG GGT GTT CTC TAT GTA GGC TCC AAA ACC AAG GAG   190
 31    G   K   T   K   E   G   V   L   Y   V   G   S   K   T   K   E    46

191   GGA GTG GTG CAT GGT GTG GCA ACA GTG GCT GAG AAG ACC AAA GAG CAA   238
 47    G   V   V   H   G   V   A   T   V   A   E   K   T   K   E   Q    62

239   GTG ACA AAT GTT GGA GGA GCA GTG GTG ACG GGT GTG ACA GCA GTA GCC   286
 63    V   T   N   V   G   G   A   V X V   T   G   V   T   A   V   A    78

287   CAG AAG ACA GTG GAG GGA GCA GGG AGC ATT GCA GCA GCC ACT GGC TTT   334
 79    Q   K   T   V   E   G   A   G   S Y I   A   A   A   T   G   F    94

335   GTC AAA AAG GAC CAG TTG GGC AAG AAT GAA GAA GGA GCC CCA CAG GAA   382
 95    V   K   K   D   Q   L   G   K   N   E   E   G   A   P   Q   E   110

383   GGA ATT CTG GAA GAT ATG CCT GTG GAT CCT GAC AAT GAG GCT TAT GAA   430
111    G   I   L   E   D   M   P   V   D   P   D   N   E   A   Y   E   126

431   ATG CCT TCT GAG GAA GGG TAT CAA GAC TAC GAA CCT GAA GCC TAA GAA   478
127    M   P   S   E   E   G   Y   Q   D   Y   E   P   E   A  **       140
```

Fig. 2A-1

```
 479  ATA TCT TTG CTC CCA GTT TCT TGA GAT CTG CTG ACA GAT GTT CCA TCC   526
 527  TGT ACA AGT GCT CAG TTC CAA TGT GCC CAG TCA TGA CAT TTC TCA AAG   574
 575  TTT TTA CAG TGT ATC TCG AAG TCT TCC ATC AGC AGT GAT TGA AGT ATC   622
 623  TGT ACC TGC CCC CAC TCA GCA TTT CGG TGC TTC CCT TTC ACT GAA GTG   670
 671  AAT ACA TGG TAG CAG GGT CTT TGT GTG CTG TGG ATT TTG TGG CTT CAA   718
 719  TCT ACG ATG TTA AAA CAA ATT AAA AAC ACC TAA GTG ACT ACC ACT TAT   766
 767  TTC TAA ATC CTC ACT ATT TTT TTG TTG CTG TTG TTC AGA AGT TGT TAG   814
 815  TGA TTT GCT ATC ATA TAT TAT AAG ATT TTT AGG TGT CTT TTA ATG ATA   862
 863  CTG TCT AAG AAT AAT GAC GTA TTG TGA AAT TTG TTA ATA TAT ATA ATA   910
 911  CTT AAA AAT ATG TGA GCA TGA AAC TAT GCA CCT ATA AAT ACT AAA TAT   958
 959  GAA ATT TTA CCA TTT TGC GAT GTG TTT TAT TCA CTT GTG TTT GTA TAT  1006
1007  AAA TGG TGA GAA TTA AAA TAA AAC GTT ATC TCA TTG CAA AAA TAT TTT  1054
1055  ATT TTT ATC CCA TCT CAC TTT AAT AAT AAA AAT CAT GCT TAT AAG CAA  1102
1103  CAT GAA TTA AGA ACT GAC ACA AAG GAC AAA AAT ATA AAG TTA TTA ATA  1150
1151  GCC ATT TGA AGA AGG AGG AAT TTT AGA AGA GGT AGA GAA AAT GGA ACA  1198
1199  TTA ACC CTA CAC TCG GAA TTC CCT GAA GCA ACA CTG CCA GAA GTG TGT  1246
1247  TTT GGT ATG CAC TGG TTC CTT AAG TGG CTG TGA TTA ATT ATT GAA AGT  1294
1295  GGG GTG TTG AAG ACC CCA ACT ACT ATT GTA GAG TGG TCT ATT TCT CCC  1342
1343  TTC AAT CCT GTC AAT GTT TGC TTT ATG TAT TTT GGG GAA CTG TTG TTT  1390
1391  GAT GTG TAT GTG TTT ATA ATT GTT ATA CAT TTT TAA TTG AGC CTT TTA  1438
1439  TTA ACA TAT ATT GTT ATT TTT GTC TCG AAA TAA TTT TTT AGT TAA AAT  1486
1487  CTA TTT TGT CTG ATA TTG GTG TGA ATG CTG TAC CTT TCT GAC AAT AAA  1534
1535  TAA TAT TCG ACC ATG AAA AAA AAA AA                               1560
```

Fig. 2A-2

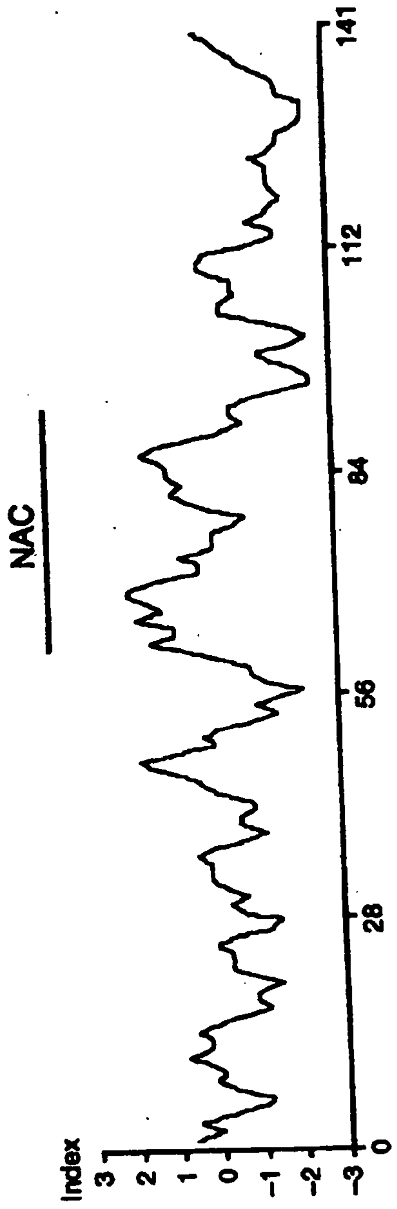


Fig. 2B

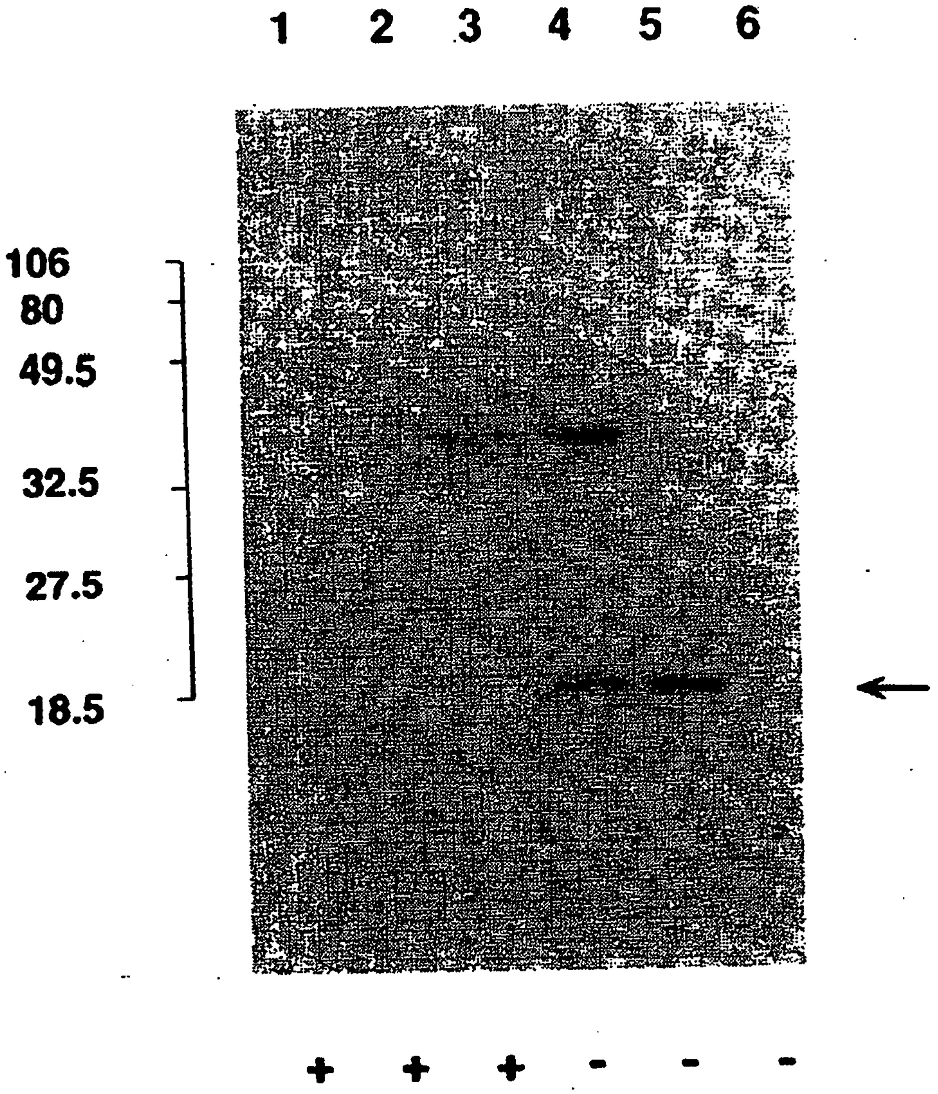

1
2
3
4
5
6
106
80
49.5
32.5
27.5
18.5
+
+
+
-
-
-

Fig. 3

10
N---KAKEGV--
21
--KTKQGV--
32
--KTKEGV--
43
--KTKEGV--
58
--KTKEQV--
80
--KTVEGA--
102
--KNEEGA---C

Fig. 4A

48
N---VVHGVATVA--
70
--VVTGVTAVA---C

Fig. 4B

```
      1        10        20        30        40        50
NACP  MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKT••KEGVVHGVATV
      :::: :: : :::::: : ::::::: ::: :::::: :::     ::
EST   MDVFKKGFSIAKEGVVGAVEKTKQGVTEAAEKTKEGVMYVGLHFFFKERIN*.....
      1        10        20        30        40        50
```

Fig. 4C

Fig. 5A

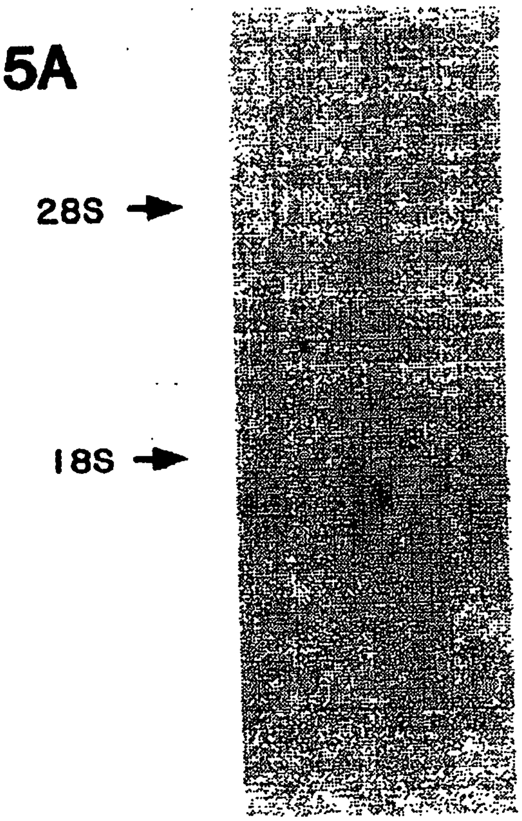
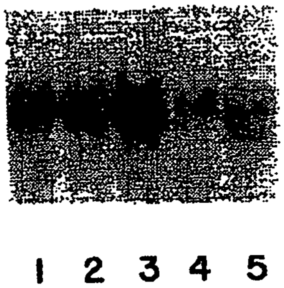


Fig. 5B



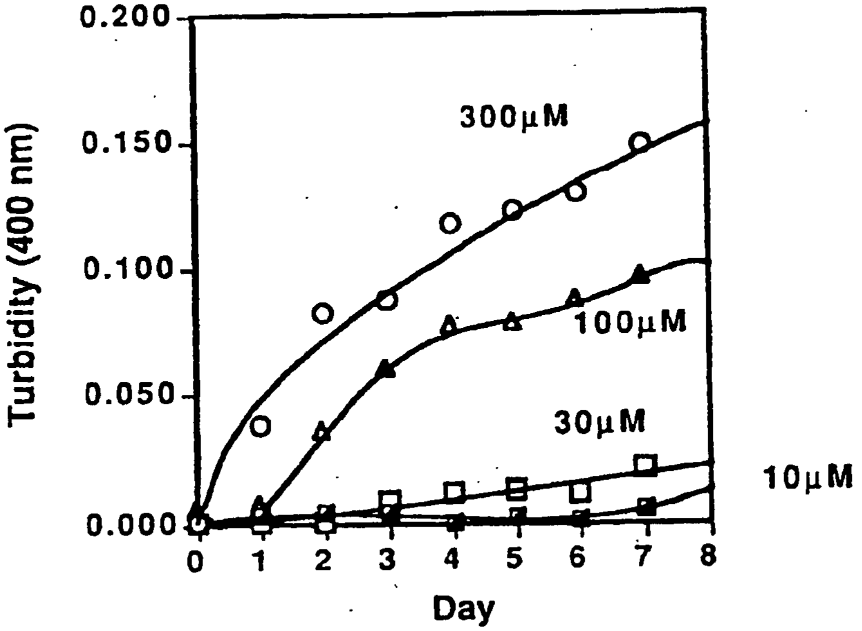

0.200
0.150
0.100
0.050
0.000
Turbidity (400 nm)
300µM
100µM
30µM
10µM
0
1
2
3
4
5
6
7
8
Day

Fig. 6A

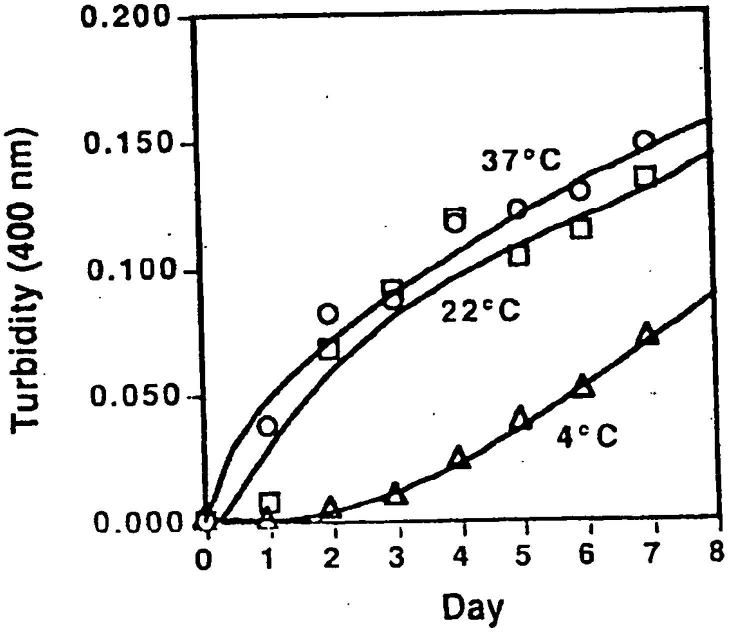

0.200
0.150
0.100
0.050
0.000
Turbidity (400 nm)
37°C
22°C
4°C
0
1
2
3
4
5
6
7
8
Day

Fig. 6B

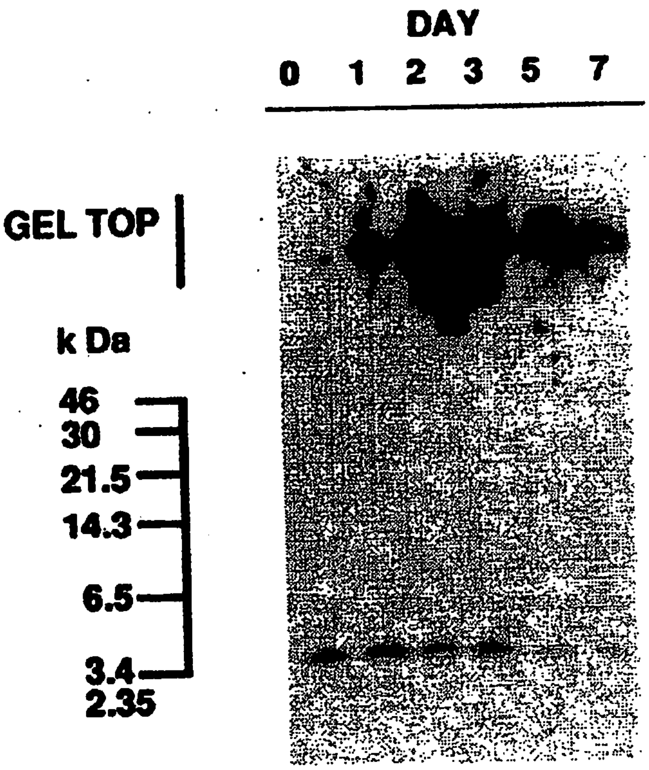

DAY
0
1
2
3
5
7
GEL TOP
k Da
46
30
21.5
14.3
6.5
3.4
2.35

Fig. 7

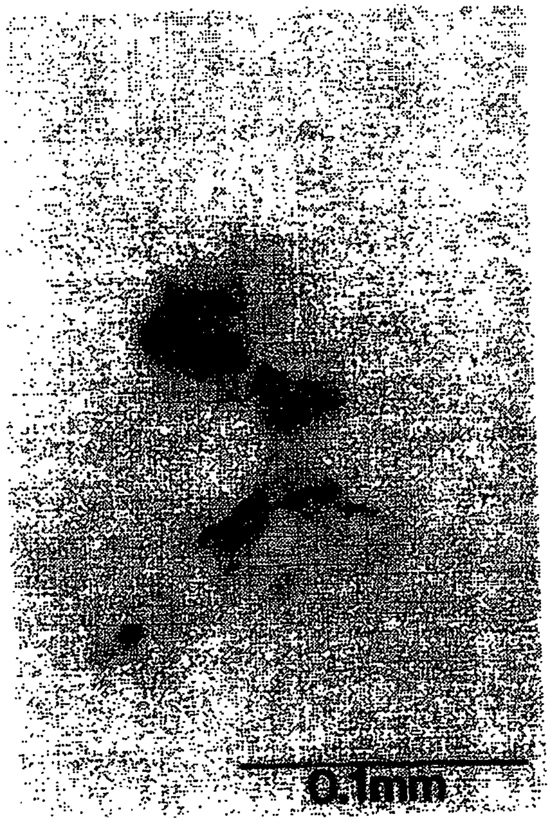


Fig. 8A

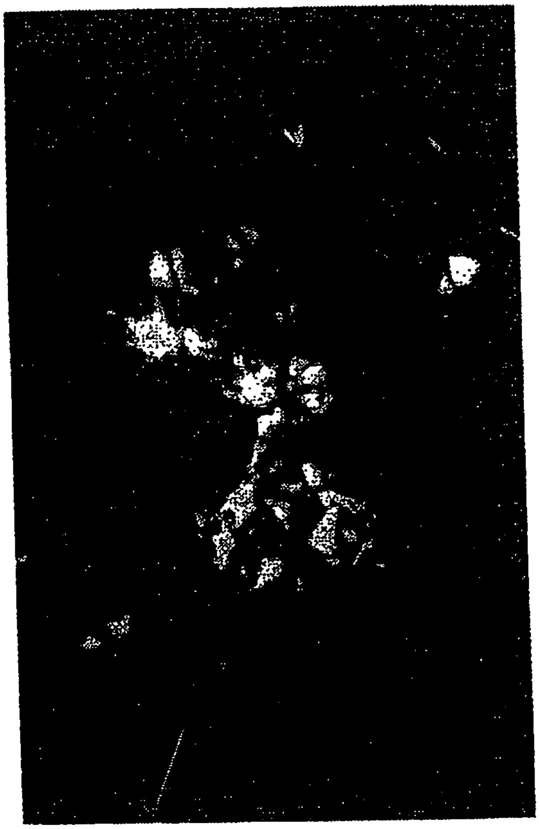


Fig. 8B

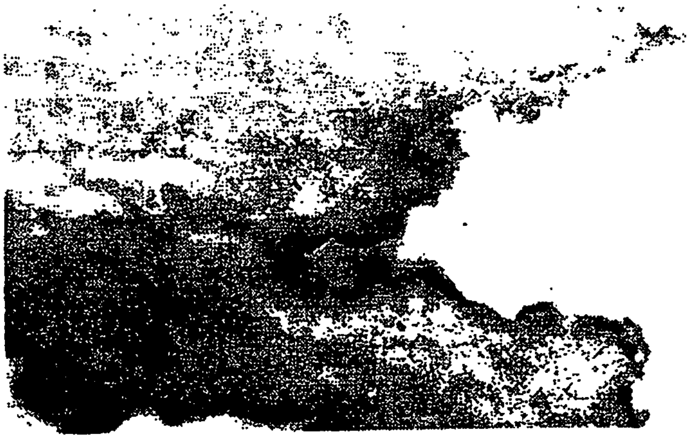


Fig. 9A

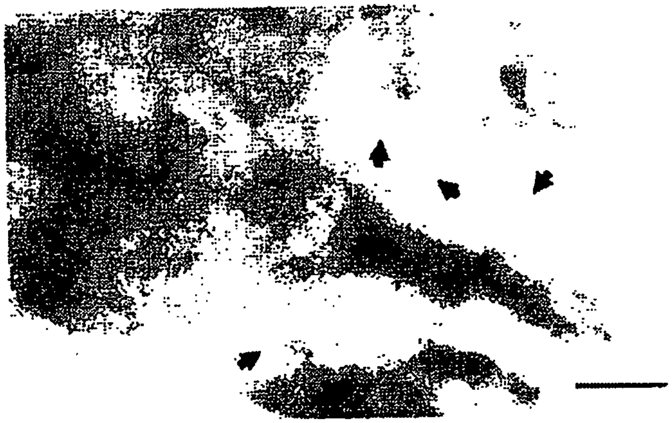


Fig. 9B

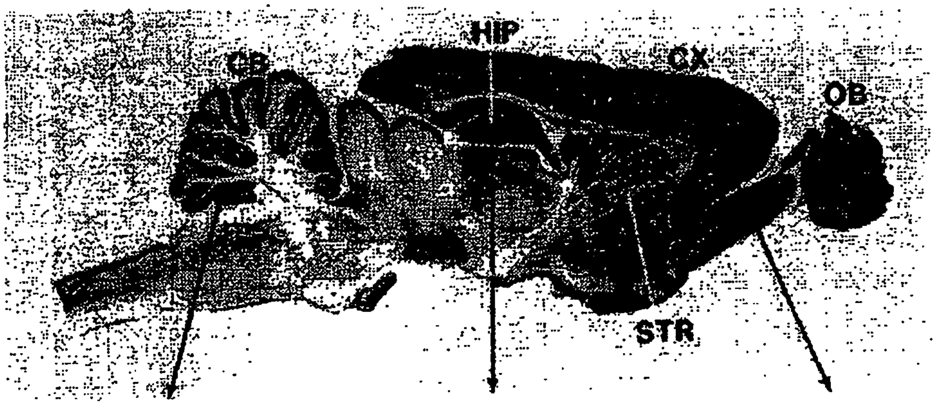

HIP
CB
CX
OB
STR

Fig. 10A

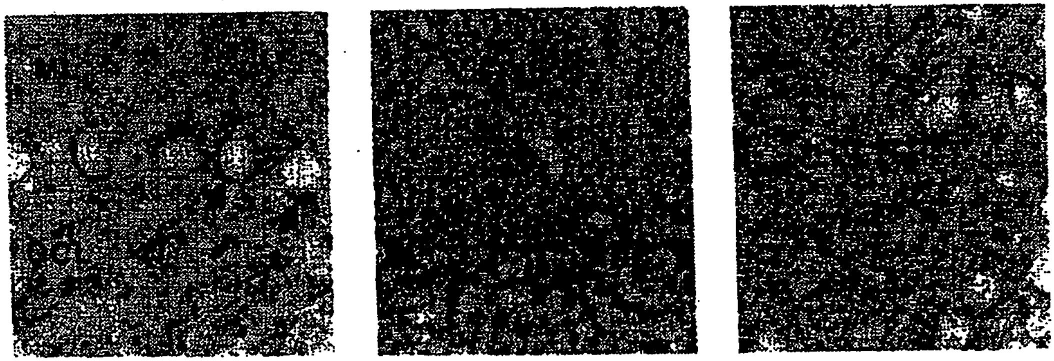


Fig. 10B Fig. 10C Fig. 10D

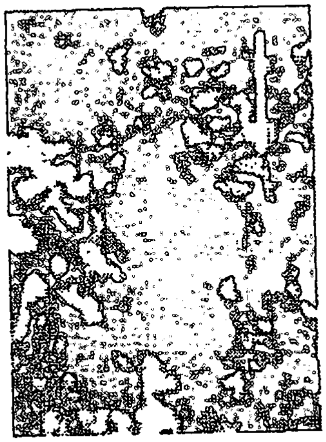


Fig. 11A

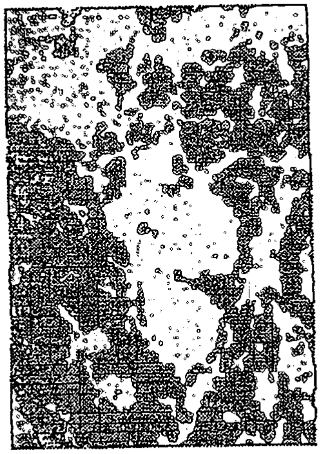


Fig. 11B

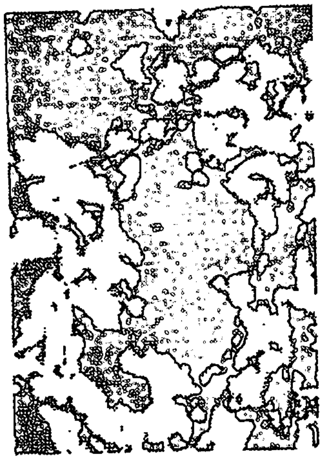


Fig. 11C

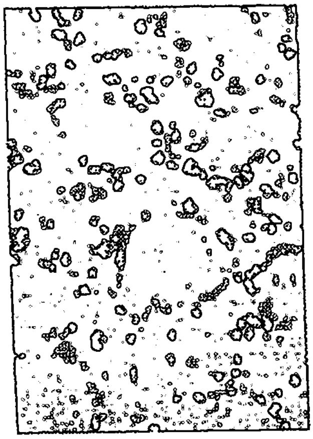


Fig. 11D

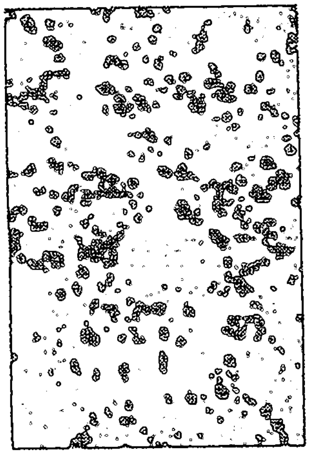


Fig. 11E

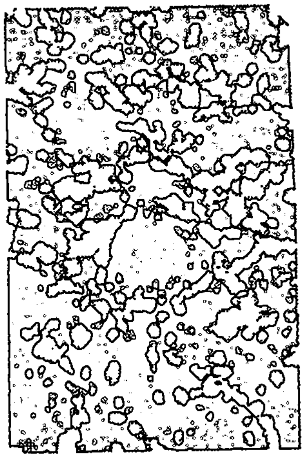


Fig. 11F

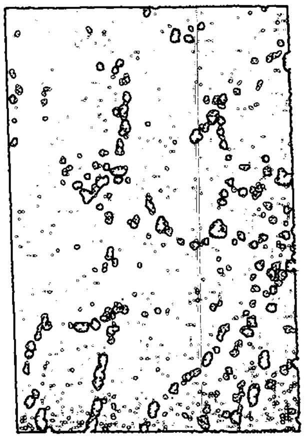


Fig. 11G

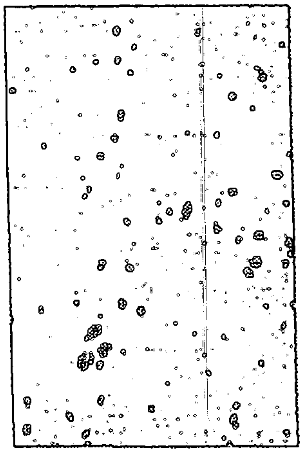


Fig. 11H

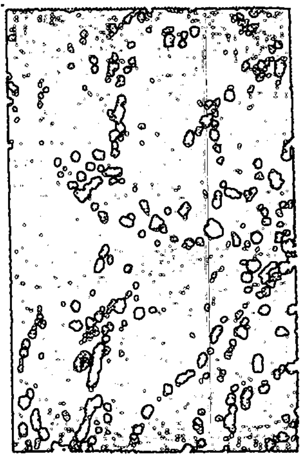


Fig. 11I

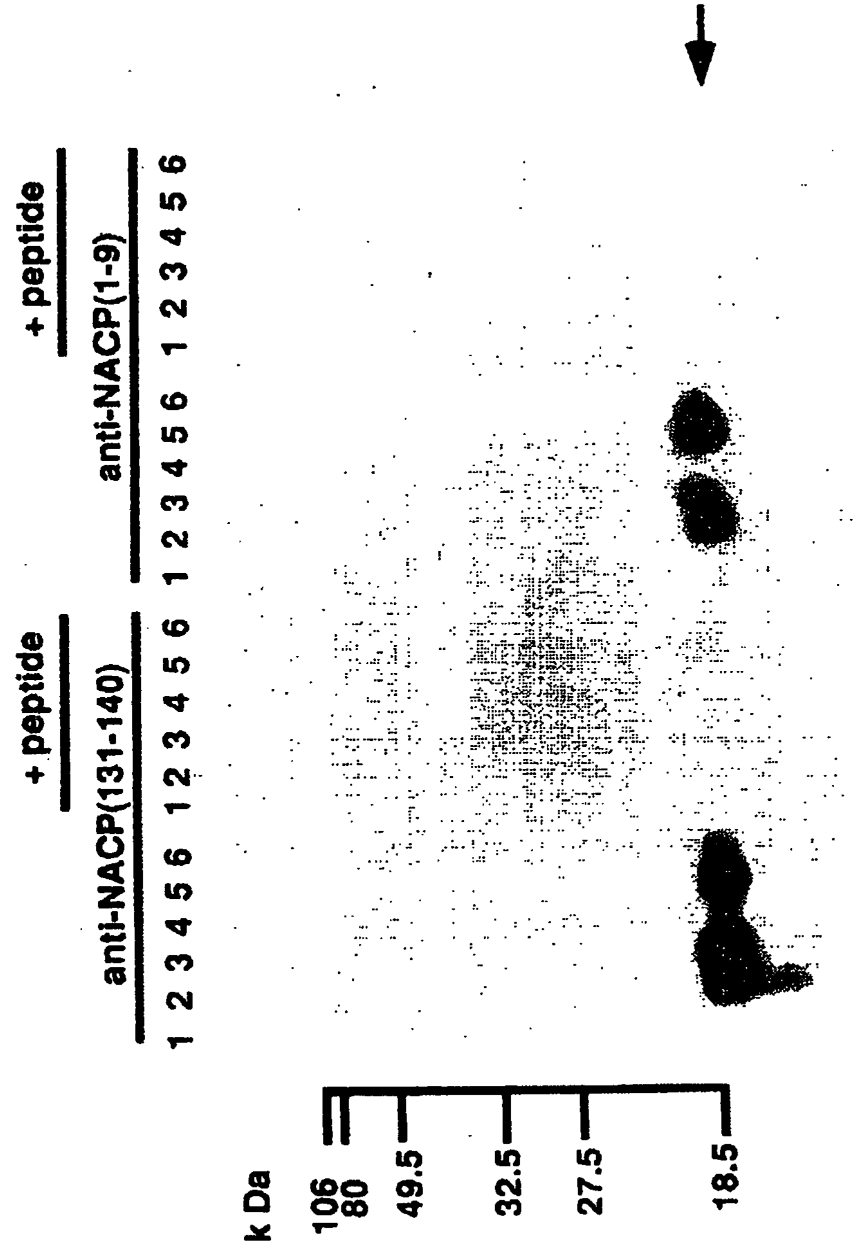

+ peptide
anti-NACP(131-140)
1 2 3 4 5 6 1 2 3 4 5 6
+ peptide
anti-NACP(1-9)
1 2 3 4 5 6 1 2 3 4 5 6
k Da
106
80
49.5
32.5
27.5
18.5

Fig. 14

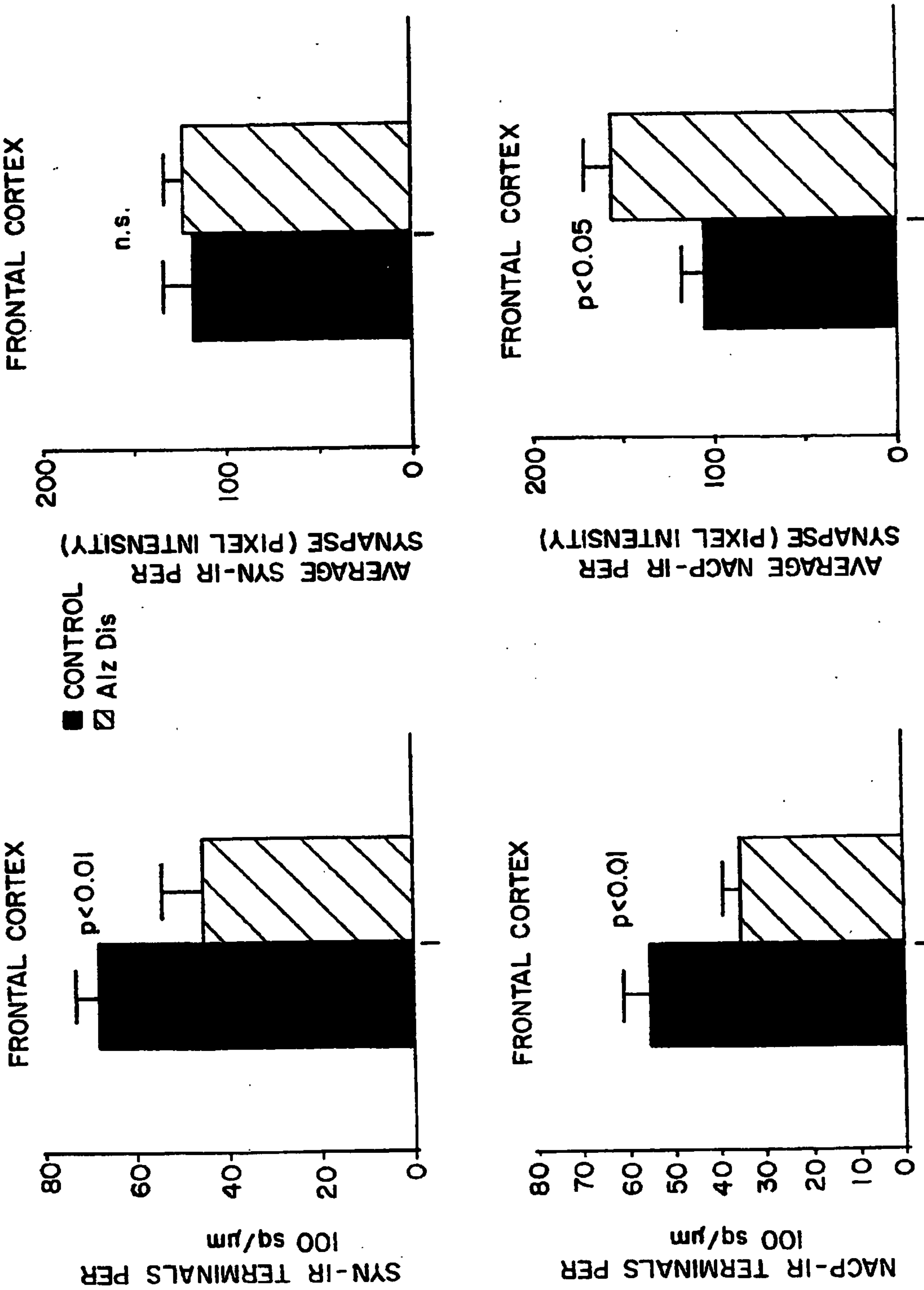

FRONTAL CORTEX
n.s.
AVERAGE SYN-IR PER SYNAPSE (PIXEL INTENSITY)
200
100
0
FRONTAL CORTEX
p<0.05
AVERAGE NACP-IR PER SYNAPSE (PIXEL INTENSITY)
200
100
0
■ CONTROL
▨ Alz Dis
FRONTAL CORTEX
p<0.01
SYN-IR TERMINALS PER 100 sq/µm
80
60
40
20
0
FRONTAL CORTEX
p<0.01
NACP-IR TERMINALS PER 100 sq/µm
80
70
60
50
40
30
20
10
0

FIG. 15

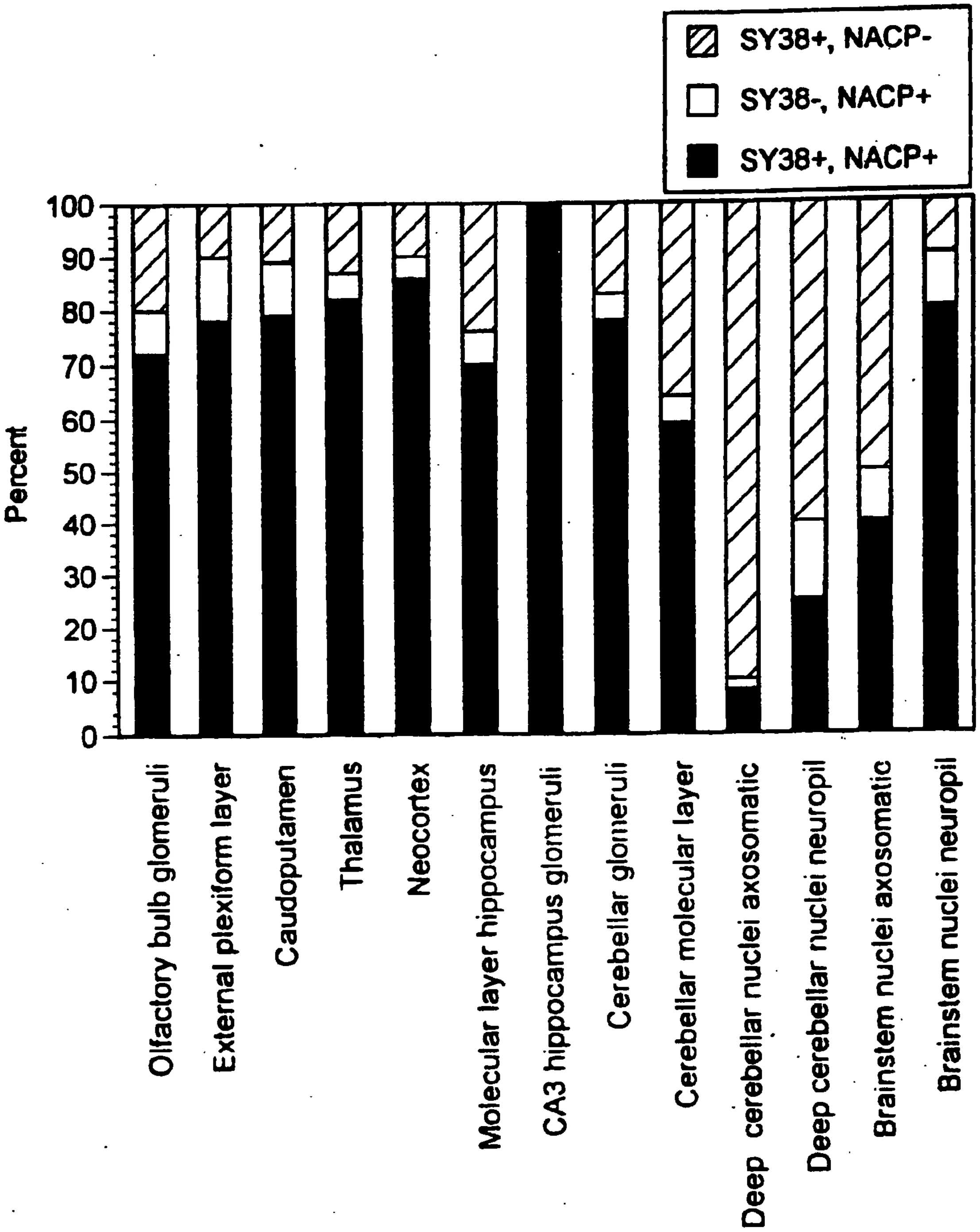

▨ SY38+, NACP-
☐ SY38-, NACP+
■ SY38+, NACP+
Percent
100
90
80
70
60
50
40
30
20
10
0
Olfactory bulb glomeruli
External plexiform layer
Caudoputamen
Thalamus
Neocortex
Molecular layer hippocampus
CA3 hippocampus glomeruli
Cerebellar glomeruli
Cerebellar molecular layer
Deep cerebellar nuclei axosomatic
Deep cerebellar nuclei neuropil
Brainstem nuclei axosomatic
Brainstem nuclei neuropil

Fig. 12

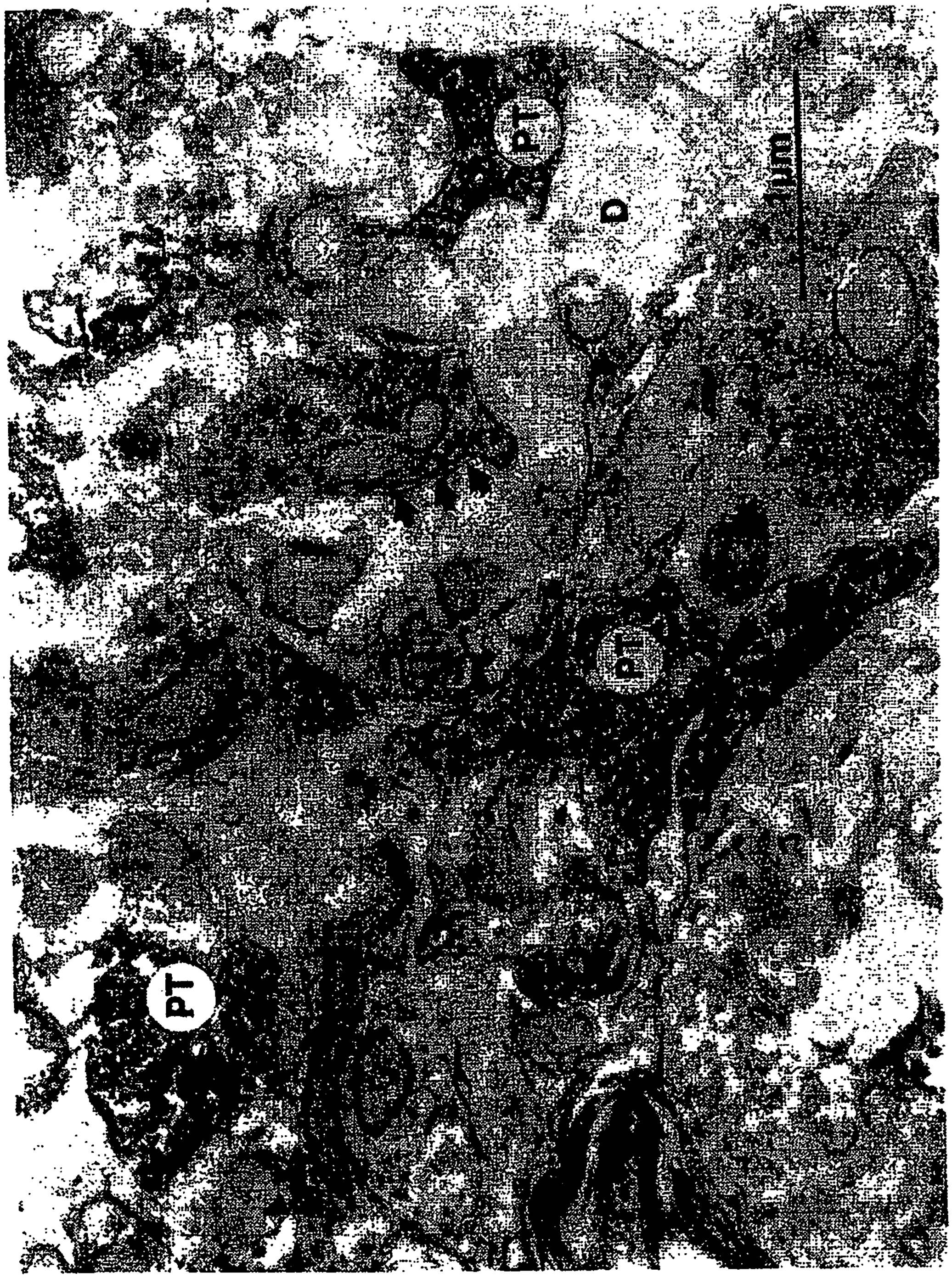
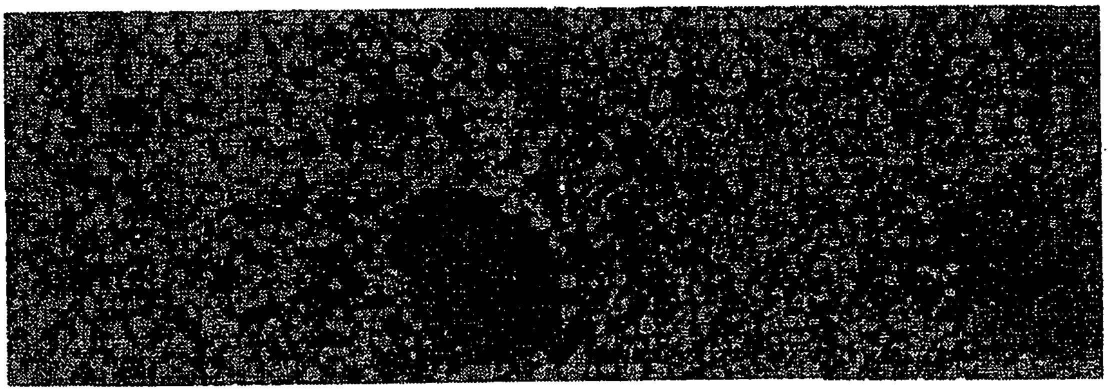

PT
D
1µm
PT
PT

Fig. 13



CONTROL

NACP

SYNAPTOPHYSIN

ALZHEIMER DISEASE

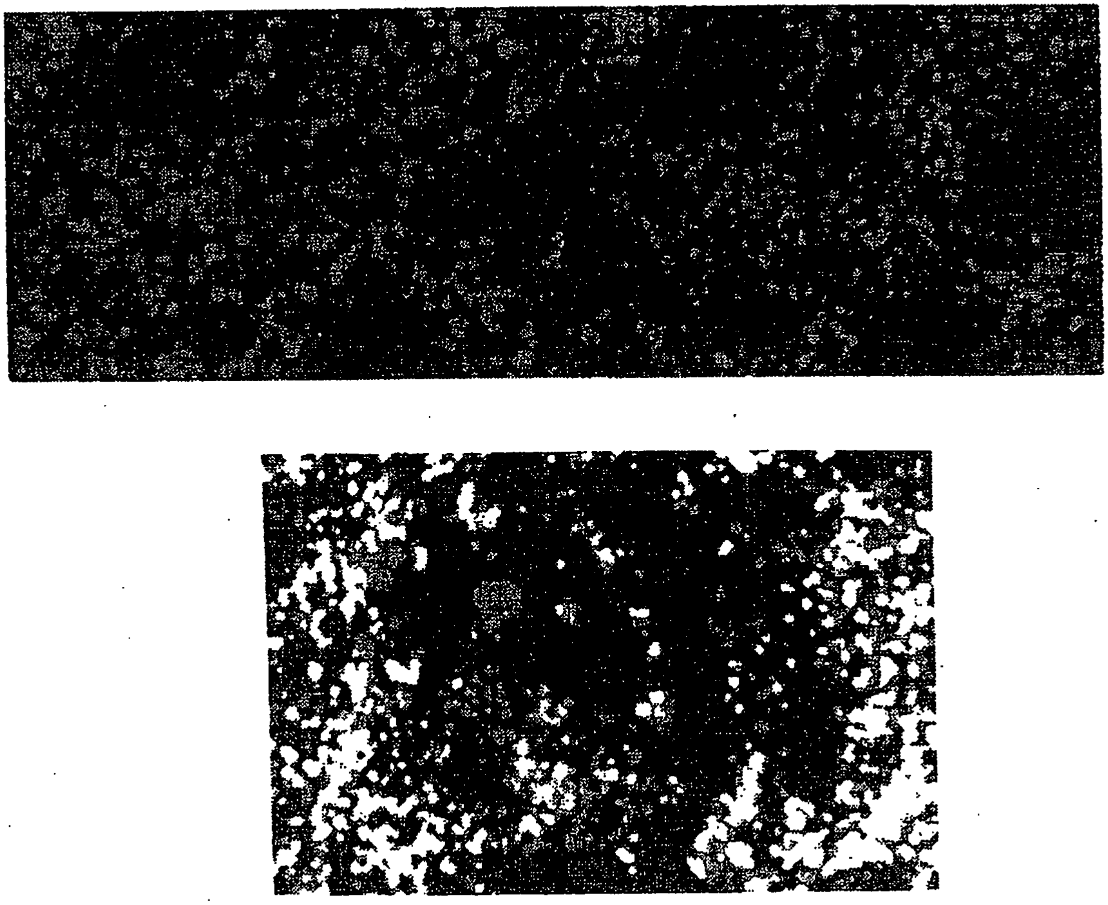
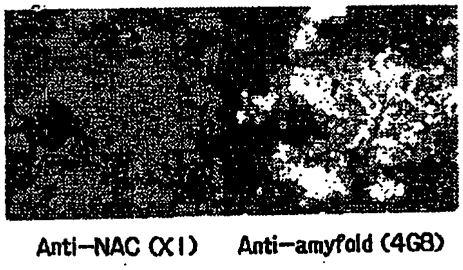


Fig. 16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Anti-amyloid (4G8)

Fig. 17A

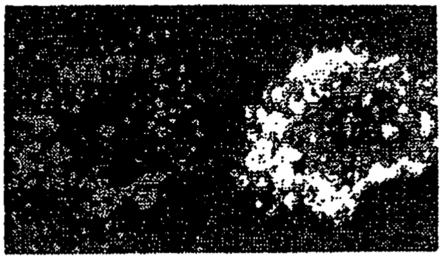

Fig. 17B

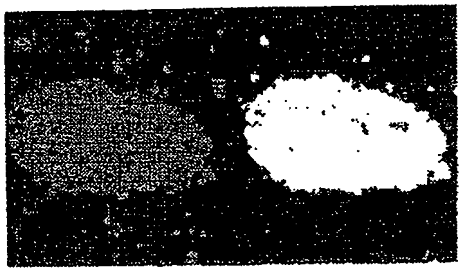

Fig. 17C

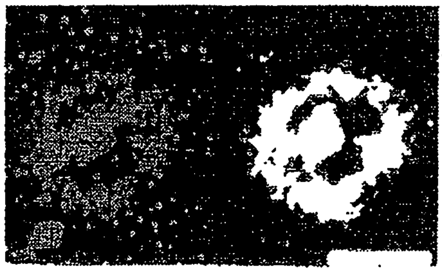

Fig. 17D

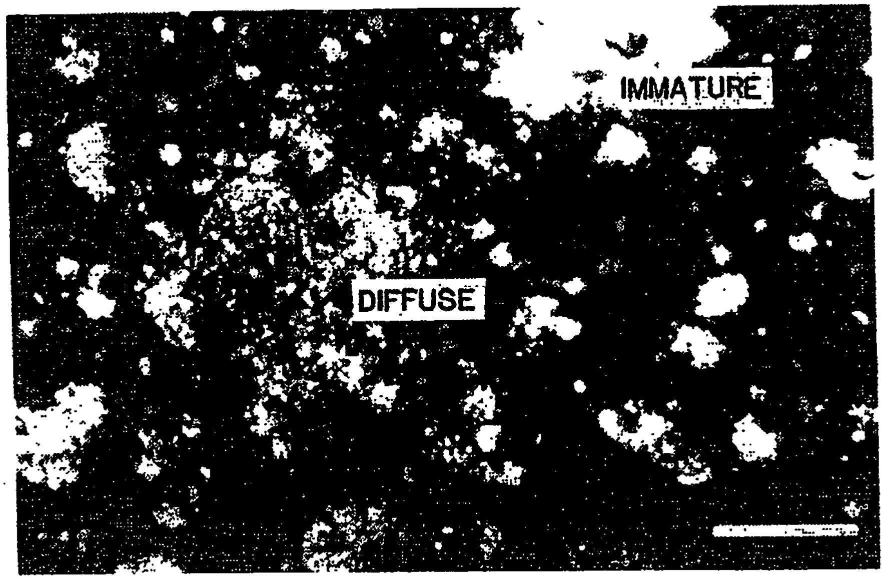
IMMATURE
DIFFUSE

Fig. 17E

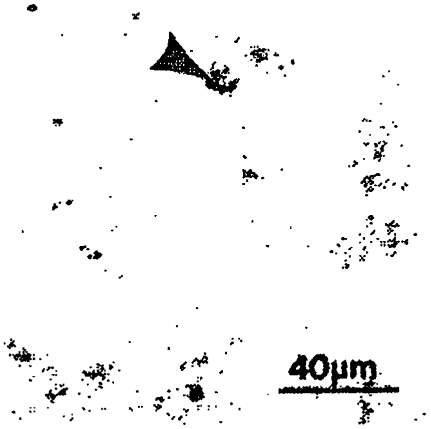

Fig. 18A

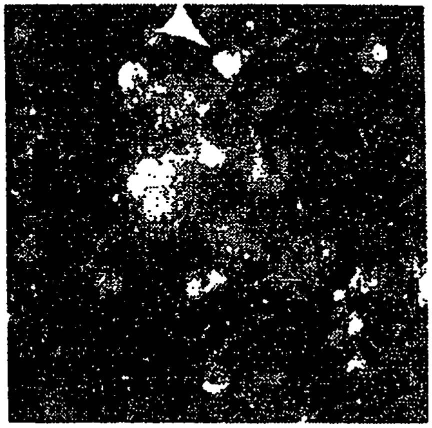

Fig. 18B

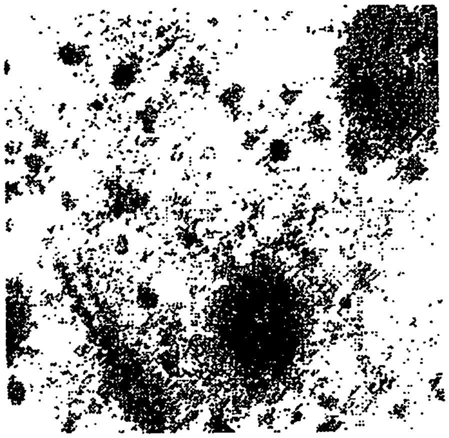

Fig. 18C

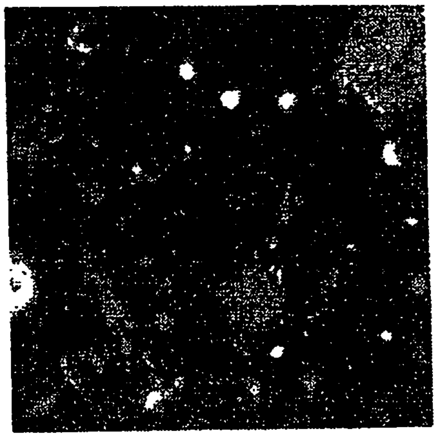

Fig. 18D

Fig. 18E

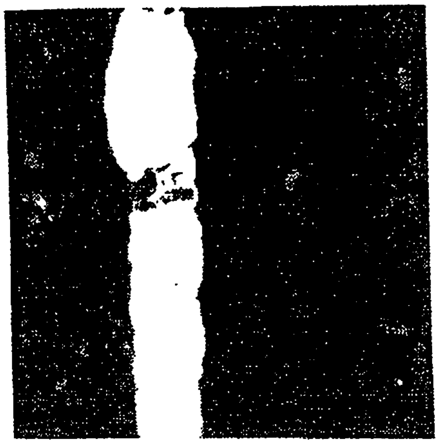

Fig. 18F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09789

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.2, 7.8, 69.1, 240.2, 252.3, 320.1; 514/2, 12; 530/300, 326, 387.1; 536/22.1, 23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Biosis, CAS Online, APS
Search terms: alzheimer?, novel amyloid component?, amyloid fibril#, prea4, protein x, protein y

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. NATL. ACAD. SCI. USA, Vol. 82, issued June 1985, Masters et al., "Amyloid plaque core protein in Alzheimer disease and Down syndrome", pages 4245-4249, see entire document.	1-92
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 120, No. 3, issued 16 May 1984, Glenner et al., "Alzheimer's Disease: Initial Report of the Purification and Characterization of a Novel Cerebrovascular Amyloid Protein", pages 885-890, see entire document.	1-92

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 09 DECEMBER 1994	Date of mailing of the international search report 19 DEC 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Hyosuk Kim Jill Warden for Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMERICAN JOURNAL OF PATHOLOGY, Vol. 141, No. 4, issued October 1992, Price et al., "Amyloidosis in Aging and Alzheimer's Disease", pages 767-772, see entire document.	1-92
X, P	PROC. NATL. ACAD. SCI. USA, Vol. 90, issued December 1993, Ueda et al., "Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease", pages 11282-11286, see entire document.	1-92

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09789

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 37/18; A61K 35/14, 38/00; C07H 17/00, 19/00, 21/00; C07K 1/00, 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00; C12N 1/20, 5/00, 15/00; C12P 21/00; C12Q 1/00, 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 7.2, 7.8, 69.1, 240.2, 252.3, 320.1; 514/2, 12; 530/300, 326, 387.1; 536/22.1, 23.1, 23.5